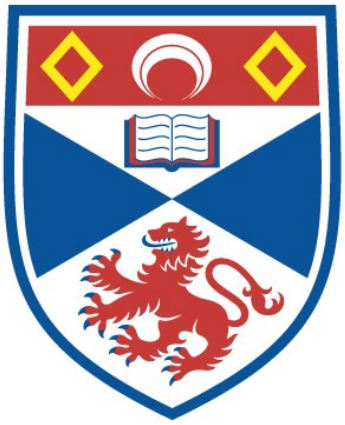
CHOLINERGIC MODULATION OF SPINAL MOTONEURONS AND LOCOMOTOR CONTROL NETWORKS IN MICE

Filipe Nascimento

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Filipe Nascimento

University of
St Andrews

This thesis is submitted in partial fulfilment for the degree of Doctor of Philosophy at the University of St Andrews

May 2018

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Contents

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LIST OF ABBREVIATIONS

5-HT - 5-hidroxitriptamine or serotonin
ACh – acetylcholine
aCSF – artificial cerebrospinal fluid
ALS - Amyotrophic Lateral Sclerosis
ChAT - choline acetyltransferase
C_m – capacitance
CPG – Central Pattern Generator
DA – dopamine
Dbx1 - developing brain homeobox protein
DREADD - Designer Receptor Exclusively Activated by Designer Drugs
EGFP - enhanced green fluorescent protein
En1 - Engrailed-1 transcription factor
Evx1 - Even-Skipped Homeobox 1
GABA - γ-Aminobutyric acid
Ia - A-type K^+ current
I_h - hyperpolarization activated current
I_{KIR} - inward rectifying K^+ currents
IN - Interneuron
Kv2.1 – voltage-gated K^+ channel 2.1
Lhx3 - LIM homeobox 3 transcription factor
mAChR – muscarinic acetylcholine receptor
mAHP – medium afterhyperpolarization phase
M-current - slowly activating voltage-regulated K^+ current
MLR - Mesencephalic Locomotor Region
MN – Motoneuron
mPSC – miniature postsynaptic current
nAChR – nicotinic acetylcholine receptor
NMDA - N-Methyl-D-aspartic acid
NMJ - neuromuscular junction
Pitx2 - Paired-like homeodomain 2
pMN - motoneuron progenitor domain
PCR – polymerase chain reaction
PSC – postsynaptic current
Sim1 - single-minded homolog 1
SK - small conductance Ca^{2+}-activated K^+ channel
vAChT – vesicular acetylcholine transporter
vGlutT2 – vesicular glutamate transporter 2
WT- wild type

ABSTRACT

Locomotion is an innate behaviour that is controlled by different areas of the central nervous system, which allow for effectiveness of movement. The spinal cord is an important centre involved in the generation and maintenance of rhythmic patterns of locomotor activity such as walking and running. Interneurons throughout the ventral horn of the spinal cord form the locomotor central pattern generator (CPG) circuit, which produces rhythmic activity responsible for hindlimb movement. Motoneurons within the lumbar region of the spinal cord innervate the leg muscles to convey rhythmic CPG output to drive appropriate muscle contractions. Intrinsic modulators, such as acetylcholine acting via M2 and M3 muscarinic receptors, regulate CPG circuitry to allow for flexibility of motor output. Using electrophysiology and genetic techniques, this work characterized the receptors involved in cholinergic modulation of locomotor networks and the role and mechanism of action of a subpopulation of genetically identified cholinergic interneurons in the lumbar region of the neonatal mouse spinal cord.

Firstly, the effects of M2 and M3 muscarinic receptors on the output of the lumbar locomotor network were characterised. Experiments in which fictive locomotor output was recorded from the ventral roots of isolated spinal cord preparations revealed that M3 muscarinic receptors are important in stabilizing the locomotor rhythm while M2 muscarinic receptor activation seems to increase the irregularity of the locomotor frequency whilst increasing the strength of the motor output. This work then explored the cellular mechanisms through which M2 and M3 muscarinic receptors modulate motoneuron output. M2 and M3 receptor activation exhibited contrasting effects on motoneuron function suggesting that there is a fine balance between the activation of

these two receptor subtypes. M2 receptor activation induces an outward current and decreases synaptic drive to motoneurons while M3 receptors are responsible for an inward current and increase in synaptic inputs to motoneurons. Despite the different effects of M2 and M3 receptor activation on synaptic drive and subthreshold properties of MNs, both M2 and M3 receptors are required for muscarine-induced increase in motoneuron output. CPG networks therefore appear to be subject to balanced cholinergic modulation mediated by M2 and M3 receptors, with the M2 subtype also being important for regulating the intensity of motor output.

Next, using Designer Receptor Exclusively Activated by Designer Drug (DREADD) technology, the impact of the activation or inhibition of a genetically identified group of cholinergic spinal interneurons that express the Paired-like homeodomain 2 (Pitx2) transcription factor was explored. Stimulation of these interneurons increased motoneuron output through the activation of M2 muscarinic receptors and subsequent modulation of Kv2.1 channels. Inhibition of $Pitx2^{+}$ interneurons during fictive locomotion decreased the amplitude of locomotor bursting. Genetic ablation of these cells confirmed that $Pitx2^{+}$ interneurons increase the strength of locomotor output by activating M2 muscarinic receptors.

Overall, this work provides new insights into the receptors and mechanisms involved in intraspinal cholinergic modulation. Furthermore, this study provides direct evidence of the mechanism through which $Pitx2^{+}$ interneurons regulate motor output. This work is not only important for advancing understanding of locomotor networks that control hindlimb locomotion, but also for dysfunction and diseases where the cholinergic system is impaired such as Spinal Cord Injury and Amyotrophic Lateral Sclerosis.

COLABORATIVE STATEMENT

Thirty ventral root recordings performed in Wild Type mice (16 with methoctramine and 14 with 4-DAMP) were done in collaboration with Lennart Spindler. Figure 4.29 was provided by Maria Mina and Laskaro Zagoraiou, Academy of Athens.

1. INTRODUCTION

1.1. Spinal Control of Movement

Movement is a mechanical feature that allows an individual to change their position or orientation towards a reference point. A variety of different movements are necessary for survival such as grasping and reaching for food and escaping from predators. Movements are complex behaviours that require a range of neuronal outputs from different regions of the nervous system. A hierarchy of diverse centres for control of movement exist within the nervous system that can adapt, program and differently execute a plurality of actions. Understanding how these networks function will ultimately contribute to our knowledge of how the central nervous system controls movement in health and disease.

The motor cortex is involved in the initiation and control of voluntary movements. The motor cortex integrates sensory information from peripheral afferents and is responsible for muscle activation, setting command for adequate muscle force and direction of movement. This brain area is also involved in the organization and programming of motor sequences. Neurons projecting through the corticospinal tract affect, directly or indirectly through descending brainstem pathways, neurons involved in the generation of muscle contraction thus controlling movement (Scott, 2004; Guertin, 2013).

The brainstem is involved in the control of posture and several autonomic functions. It also integrates sensory information originating from visual, somatosensory and vestibular inputs which can be translated into changes in the modulation of motor output (Kim *et al.*, 2017; Mathews *et al.*, 2017). The Mesencephalic Locomotor Region (MLR) is located in the brainstem and is involved in the initiation of motor drive (Jordan *et al.*, 2008). Several neurotransmitters can be released after MLR activation,

such as the classical excitatory amino acid glutamate that can trigger functional changes in spinal circuits sufficient to produce locomotor activity (Hochman et al. 1994; Kim et al. 2017; Jordan et al. 2008; Cazalets et al. 1992).

The spinal cord is an important centre for the control of movement, being able to provide enough output for rhythmic movements without the need for information from higher brain areas. This was first suggested more than 100 years ago in experiments performed in the spinalised cat. After cutting the communication between the brain and the spinal cord and dorsal roots to remove sensory input, Brown (1911) was able to detect muscle contraction followed by relaxation along with alternation between respective extensor and flexor muscles in the cat hindlimb (Brown, 1911). This idea, which was highly discussed during the beginning of the 20th century (see Stuart & Hultborn 2008), became the first evidence that central circuits can produce rhythmic behaviours, these networks being known today as Central Pattern Generators (CPGs).

Different CPGs have been described in a variety of systems and animals, differing in their properties for the generation of rhythmic activity. In systems such as the lobster pyloric and leach rhythm generating networks, intrinsically oscillating pacemaker neurons can generate rhythmic activity. In more complex networks such as mammalian spinal locomotor networks, the generation of rhythmic patterns is dependent on the interaction between different types of neurons. Reciprocal inhibition underlies oscillatory-type firing where inhibitory interneurons project to contralateral excitatory neurons, thus acting as "half centre oscillators" where one half centre inhibits its contralateral counterpart (Marder and Bucher, 2001).

These CPG networks can be modulated by either descending or by local modulators that can affect the rhythm generating properties of neurons allowing for a

variety of adaptable behaviours (Miles and Sillar, 2011). The work presented in this thesis focussed on CPG networks in the spinal cord responsible for mammalian hindlimb locomotion and their fine-tuning by local modulators.

1.2. Mammalian spinal locomotor Central Pattern Generator

The exact organization and function of the neurons responsible for the generation of rhythmicity in the mammalian spinal cord remain unclear. Different *in vivo* and *in vitro* preparations have been used to study locomotor circuits. *In vivo* insights from the adult mammalian CPG were drawn from experiments using decerebrated animals in which electrical activation of brainstem locomotor regions or pharmacological excitation elicited fictive locomotion that was recorded from hindlimbs through electroneurograms. Fictive locomotion is considered the output measured from muscle or ventral roots in the absence of muscle contraction (e.g. muscles removed or paralyzed) but that would ultimately lead to limb movement. Early studies were performed in the cat (Orlovskiĭ *et al.*, 1966; Jankowska *et al.*, 1967; Grillner and Zangger, 1979) and later utilised rabbit (Viala and Buser, 1971), rat (Kinjo *et al.*, 1990; Bem *et al.*, 1993), mice (Meehan *et al.*, 2012) and also marmoset monkey (Fedirchuk *et al.*, 1998). *In vitro* spinal cord preparations from neonatal rodents, which allow pharmacological activation and manipulation of the locomotor CPG and recording of neuronal activity, have been used extensively to study network organisation (Kudo and Yamada, 1987; Smith and Feldman, 1987). In isolated spinal cord preparations from neonatal mice it is possible to evoke rhythmic activity, which when recorded from ventral nerve roots resembles the pattern of walking or running in the intact animal, with alternation between bursts of activity recorded from nerves that innervate

functional antagonist (right/left and flexor/extensor) muscle groups (Jiang *et al.*, 1999). Both *in vivo* and *in vitro* preparations have proven to be important tools for advancing the understanding of the roles of different types of neurons that are part of the spinal locomotor circuitry.

1.2.1. Genetic diversity in spinal neurons

To better understand how mammalian spinal circuits generate rhythmicity, genetic tools have been used to elucidate the diversity of populations of neurons within the spinal cord that contribute to the generation and appropriate assembling of motor output. Spinal neurons can be traced based on the restricted expression of specific developmental transcription factors. This approach has proven useful to better understand the different genetically identifiable neurons that comprise the motor networks and are responsible for shaping rhythmic outputs.

It is during development that different groups of neurons start to arise with interneurons (INs) responsible for sensory input being differentiated dorsally while motoneurons (MNs) and INs involved in the generation of motor output being aligned ventrally. The differentiation of each neuronal subtype in the spinal cord is based on signalling gradients that establish a grid-like set of positional cues (Jessell, 2000) allowing a spatial division and distinction of 11 neuronal progenitor domains. From these, 6 progenitor domains (dl6, V0, V1, V2, V3 and MNs) are thought to produce neurons that are part of the locomotor circuitry while the other 5 (dl1, dl2, dl3, dl4 and dl5) contribute to sensory networks. Specific transcription factors involved in the differentiation of neurons from progenitor domains are used to identify distinct populations that may be divided further, into more specific subpopulations (Goulding,

2009). Locomotor networks require a high-level of organisation that will produce coordination between right and left sides as well as between antagonistic muscles (i.e. extensor *vs* flexor). The interplay between the different genetically identifiable populations of CPG INs allows for effectiveness and flexibility of locomotor pattern.

The developing brain homeobox protein 1 (Dbx-1) is the transcription factor that controls the fate of the V0 population of INs that displays a critical role in right-left burst alternation (Lanuza *et al.,* 2004). This population can be subdivided into $V0_V$ (ventral) and $V0_D$ (dorsal) INs according to their migration pattern during development. The V0v INs are glutamatergic and their identity is consolidated by the expression of Even-Skipped Homeobox 1 (Evx1), whereas the $V0_D$ population release γ-aminobutyric acid (GABA) or glycine (Moran-Rivard *et al.,* 2001; Pierani *et al.,* 2001). Less than 10% of the V0 neurons give rise to a subpopulation of INs identified by the expression of the Paired-like homeodomain 2 transcription factor (Pitx2). These can be subdivided into cholinergic and glutamatergic subgroups with the former being recruited during locomotion in a task-dependent manner (Zagoraiou *et al.,* 2009).

The V1 IN population can be identified by the expression of the transcription factor Engrailed-1 (En1). V1 INs include Ia inhibitory INs and Renshaw cells (Alvarez *et al.,* 2005). Ia inhibitory INs are mostly innervated by sensory afferents and are responsible for reciprocal inhibition to antagonistic MN pools whereas Renshaw cells are excited by MN axon collaterals and inhibit MN firing thus being involved in recurrent inhibition of synergistic MN pools (Renshaw, 1946; Eccles *et al.,* 1954). The V1 population can be further fractionated into more specific subsets based on a combinatory expression of different transcription factors which give rise to groups of INs with distinctive physiology and anatomical properties (Bikoff *et al.,* 2016). V1 INs

project to ipsilateral INs as well as MNs and seem to be involved in controlling the duration of the locomotor step cycle, thus being essential for fast locomotor output (Gosgnach *et al.*, 2006).

V2 INs project intersegmentally and ipsilaterally in the mammalian spinal cord. They express the transcription factor LIM homeobox 3 (Lhx3) and can give rise to 3 distinct subpopulations (V2a, V2b and V2c). Each subgroup can be traced with a specific genetic marker. The identity of the excitatory V2a INs is consolidated by the Chx10 transcription factor and this subpopulation is involved in the control of right-left alternation (Crone *et al.*, 2008; Clovis *et al.*, 2016). Ablation of inhibitory V2b INs, that express GATA binding protein 2/2 (Gata2/3), compromises alternation between reciprocal extensor and flexor muscles (Zhang *et al.*, 2014). V2c interneurons are known to derive from the same progenitor domain as V2a and V2b subpopulations but their exact role in CPG networks is still unclear (Panayi *et al.*, 2010).

The V3 IN subgroup is involved in fine-tuning the robustness of movement. V3 INs can be genetically traced by the single-minded homolog 1 (Sim1) transcription factor (Zhang *et al.*, 2008). These INs can be further divided into ventral and dorsal subpopulations, according to their distribution pattern, which exhibit different functional properties. Ventral V3 INs have a more spatially restricted branching and generate intense tonic firing, whereas dorsal V3 INs have a more complex arborization and fire at low frequencies (Borowska *et al.*, 2013).

The dI6 interneurons settle in the ventral region of the spinal cord close to the central canal of the spinal cord and can display two different functional phenotypes that were shown to be rhythmically active, highlighting a possible role in rhythm generation (Dyck *et al.*, 2012).

Hb9 INs are a group of mostly glutamatergic spinal neurons whose embryonic origin is not fully understood. These interneurons do not affect pattern generation since they do not seem to be necessary for adequate right-left or extensor-flexor phasing, however, they do seem to be involved in controlling the frequency of locomotor activity (Caldeira *et al.*, 2017).

Spinal MNs are the 'final common pathway' (Sherrington, 1906) in the spinal cord for initiation of movement. MNs originate from the motoneuron progenitor domain (pMN) and are located in the ventral horn, most specifically in lamina IX. They release acetylcholine (ACh) at the neuromuscular junction (NMJ) which ultimately leads to muscle fibre contraction. Neurons responsible for the innervation of visceral muscles (e.g. cardiac muscle, gut) will not be addressed in this work and thus the term "MN" will only refer to spinal neurons that project from the ventral horn and synapse onto skeletal muscle fibres. MNs receive a variety of motor and sensory-motor inputs from INs that contribute to the refinement of movement. There are 3 subtypes of MNs: (i) alpha (ii) beta and (iii) gamma MNs. Gamma MNs are important in adjusting the sensitivity of muscle spindles, which are sensory organs within the body of a muscle that detect changes in muscle length and control stretch responsiveness to prevent overstretching and maintain muscle tone. Alpha and beta MNs are responsible for the generation and maintenance of muscle contraction. Alpha MNs have large cell bodies and form synapses with extrafusal muscle fibres thus initiating skeletal muscle contraction. Beta MNs are smaller than alpha MNs and innervate both extrafusal and intrafusal fibres thus also adjusting the stretch responsiveness at the muscle spindle (Stifani, 2014).

Despite its complexity, the mammalian CPG exhibits some degree of conservation of the organisation that is present in other systems. The generation of rhythmicity by the crustacean stomastogastric ganglion (Marder and Bucher, 2001), lamprey (Ekeberg and Grillner, 1999) and Xenopus tadpole swimming networks (Ekeberg and Grillner, 1999), Drosophila larvae crawling circuit (Wystrach *et al.*, 2016) and leech heartbeat network (Weaver *et al.*, 2010) involves reciprocal inhibition that secures coordination and adequate oscillatory patterns. In the mammalian spinal cord, reciprocal inhibition is important for the generation of right-left as well as extensor-flexor alternation, which sets the stage for appropriate locomotor pattern. Besides the diversity in neuronal subtypes (figure 1.1), modulation of spinal networks can account for adaptable changes to network output, which are required to suit varying biomechanical and behavioural demands.

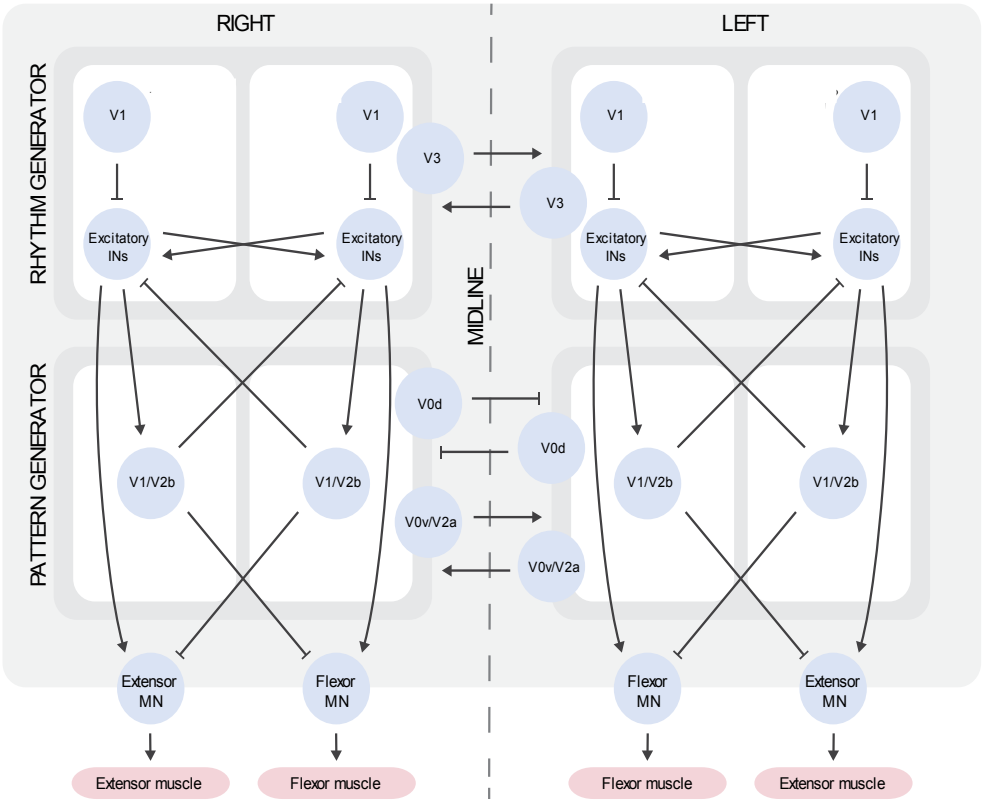


Figure 1.1 – schematic of the functional organization of the different subtypes of INs that comprise the mammalian CPG. Locomotor networks comprise a bilateral network with extensor and flexor, pattern and rhythm generating modules. Populations of genetically identified INs (blue circles) interact with each other through excitation (arrows) or inhibition (bars) to generate and sustain adequate locomotor behaviour. Adapted from Acton & Miles 2017.

1.3. Neuromodulation of spinal locomotor networks

Different locomotor behaviours are necessary for survival in mammals. Change in speed is required for a prey to escape from a predator, climbing a tree might be crucial to search for food and crawling might be necessary to shelter from adverse environmental conditions. In order to respond to these variable locomotor demands, the CPG needs to adapt and change motor output. Neuromodulation can alter synaptic function and the intrinsic properties of spinal neurons leading to changes in the speed, coordination or strength of rhythmic motor output. Modulators can arise from higher brain areas (extrinsic modulators) or from sources within the spinal cord (intrinsic or local modulators) (Miles and Sillar, 2011).

1.3.1. Extrinsic neuromodulators

Projections from the brainstem, cortex and other supraspinal nuclei can affect spinal locomotor output. The sources and roles of three of the most studied extrinsic modulators: 5-hidroxitriptamine or serotonin (5-HT), noradrenalin (NA) and dopamine (DA), are discussed below.

1.3.1.1. Serotonin

The first insights into the role of 5-HT in CPG networks came from studies in the lamprey spinal cord (Harris-Warrick and Cohen, 1985) and the stomatogastric ganglion of the lobster (Marder and Eisen, 1984), which showed that 5-HT affected rhythmic motor behaviours. In mammals, 5-HT originates from descending pathways that project from the raphei nuclei in the brainstem (Lakke, 1997). Stimulation of serotonergic neurons from the parapyramidal region of the mid-medulla can elicit

rhythmic locomotor-like activity in the isolated spinal cord of the rat (Liu and Jordan, 2005). In the rat it has been shown that 5-HT can initiate and modulate locomotor output since 5-HT-containing descending INs were activated after MLR stimulation and were able to produce locomotor rhythm through the activation of 5-HT_7 receptors (Cabaj *et al.*, 2017). In comparison, in isolated spinal cords, 5-HT_2 antagonists were shown to be able to block 5-HT induced patterns of activity (Cazalets *et al.*, 1992). On the other hand, in N-Methyl-D-aspartic acid (NMDA)-induced fictive locomotion, activation of the 5-HT_1 receptor can have an inhibitory effect (Beato and Nistri, 1998). Therefore 5-HT might have both excitatory and inhibitory actions through the activation of 5-HT_7/5-HT_2 and 5-HT_1 receptors, respectively. At the single cell level 5-HT depolarizes spinal INs and MNs reducing action potential threshold (Elliott and Wallis, 1992; Fedirchuk and Dai, 2004). In commissural INs, 5-HT increases N-, P/Q and L-type Ca^{2+} currents (Abbinanti & Harris-Warrick 2012; Abbinanti et al. 2012). The variety of mechanisms by which 5-HT shapes locomotor network excitability suggests that this transmitter system has a powerful modulatory influence on spinal circuits involved in the control of movement.

1.3.1.2. Noradrenalin

Neurons projecting from the locus ceruleus of the brainstem to the spinal cord are the main sources of NA (Nygren and Olson, 1977). NA can induce episodes of hindlimb locomotion in spinalised cats (Barbeau and Rossignol, 1991). In neonatal rat *in vitro* spinal cord preparations, NA can elicit slow patterns of right-left alternating motor activity, which were dependent on α1-adrenoreceptors. During NMDA-induced locomotion, activation of α2 and β adrenoreceptors slows down the rhythm while the α1

subtype increases the frequency (Sqalli-Houssain and Cazalets, 2000). In the neonatal mouse, electrical stimulation of the cauda-equina can evoke a regular alternating rhythm that can be slowed down by α1 agonists, which increase burst duration, or silenced by α2 agonists (Gordon and Whelan, 2006). These effects might reflect the variety of different cellular mechanisms that can be triggered by activation of adrenergic receptors, which have a widespread distribution in the spinal cord (Noga *et al.*, 2009). Activation of α1-adrenoreceptors decreases the medium afterhyperpolarization phase (mAHP), increases the total persistent inward current and depolarizes cat MNs (Lee and Heckman, 1999). Meanwhile, exogenous NA decreases inward rectifying K^+ currents (I_{KIR}) in neonatal rat MNs (Maylis Tartas *et al.*, 2010). In ventral horn INs, activation of the noradrenergic system through brainstem stimulation can hyperpolarize the voltage threshold, which was suggested to contribute to a monoaminergic mechanism for initiation of locomotion and facilitation of spinal reflexes (Fedirchuk and Dai, 2004). The actions of NA on the locomotor network are variable and underlie a strong modulation of the mammalian spinal motor circuitry.

1.3.1.3. Dopamine

The first studies on the role of dopaminergic modulation of locomotor circuits came from experiments in the spinalised cat where application of L-DOPA could modulate reflex circuits and promote locomotor activity (Jankowska *et al.*, 1967). The main sources of spinal DA in mammals were later shown to derive from projections originating from the A11 nuclei of the posterior hypothalamus (Skagerberg and Lindvall, 1985). These descending projections act on the excitatory D_1-like (D1 and D5) and inhibitory D_2-like (D2, D3 and D4) families of DA receptors, which are known to

be widely distributed throughout the lumbar spinal cord (Zhu *et al.*, 2007). D_1-like receptors are mostly expressed in the ventral horn where CPG neurons are located while D_2-like receptors are present in the dorsal horn and might be involved in nociception (Zhu *et al.*, 2007). DA is released during locomotion (Gerin and Privat, 1998) and can elicit fictive locomotor-like rhythmic bursts in the cat (Kiehn and Kjaerulff, 1996), rat (Kiehn and Kjaerulff, 1996) but not in mice (Sharples and Whelan, 2017). In the neonatal mouse spinal cord, DA seems to play a role in shaping drug-induced locomotor output by reducing the frequency, increasing the amplitude and contributing to burst regularity. This action is dependent on the excitation state of the network (Sharples *et al.*, 2014; Sharples and Whelan, 2017). The robust modulatory actions of DA reflect a variety of cellular mechanisms triggered by the activation of dopamine receptors on CPG neurons and MNs. In the neonatal mouse spinal cord, DA has been shown to increase MN excitability by decreasing first spike latency and the mAHP. These effects were attributed to the negative regulation of A-type K^+current (Ia) and the small conductance Ca^{2+}-activated K^+channel (SK) along with an increase in glutamatergic inputs to MNs (Han *et al.*, 2007). The increase in glutamatergic inputs through AMPA receptor activation in MNs is mediated by D1 receptors (Han and Whelan, 2009). On a particular population of CPG neurons - Hb9 INs – DA increased their oscillatory activity which resulted in increased excitation (Han *et al.*, 2007). DA has been recently described as a positive modulator of the Na^+/ K^+ ATPase, which underlies an ultra-slow AHP (usAHP) in mouse MNs and is responsible for activity-dependent changes in active locomotor networks (Picton *et al.*, 2017). DA is therefore an important extrinsic modulator that contributes to the shaping of rhythmic motor output by acting on both MNs and CPG INs.

1.3.2. Intrinsic modulators

Local or intrinsic modulators arise from within the spinal cord and contribute to functional changes in CPG networks. They can help to adjust the speed, duration, coordination and strength of the motor output depending on the type of neuromodulator that is being released (Miles and Sillar, 2011).

Classic neurotransmitters that convey motor commands via activation of fast, ionotropic receptors can also act as modulators of spinal function. Glutamatergic INs are present in the spinal cord and are part of an important excitatory drive within the CPG network (Goulding, 2009). Intrinsic glutamate can act on group 1 metabotropic glutamate receptors which results in a decrease in motor output by reducing fast inactivating Na^+ currents in mouse MNs (Iwagaki and Miles, 2011). GABA that arises from a variety of inhibitory INs in the spinal cord has been shown to decrease excitatory synaptic drive to MNs ultimately resulting in reduced firing and reduced burst amplitude (Bertrand, 1999). ACh can be released from dorsal and ventral horn INs (Oguz Kayaalp and Neff, 1980; Gillberg *et al.*, 1988) and can increase neuronal excitability to affect locomotor output in rats (Kiehn *et al.*, 1996; Jordan *et al.*, 2008).

Other molecules are also known to fine-tune CPG function. Adenosine, derived from astrocytes, acts on inhibitory A1 adenosine receptors located on CPG INs to modulate the frequency of drug-induced locomotor activity (Witts *et al.*, 2012, 2015; Acton and Miles, 2017). Nitric oxide, which is presumably released by both dorsal and lamina X INs, has been shown to modulate the timing and intensity of fictive motor output (Foster *et al.*, 2014). Peptides and peptide receptors are present in both dorsal and ventral horns of the spinal cord indicating the presence of an endogenous

peptidergic modulation of spinal circuits (Heuer *et al.*, 2000; X. Liu *et al.*, 2003). Different neuropeptides have been shown to promote modulation of rhythmic output in the neonatal mouse spinal locomotor network (Barriere *et al.*, 2005).

An important factor to consider when studying neuromodulation is that one modulator may change the role of another modulatory system. This second-order effect, known as metamodulation, can allow one modulator to have an excitatory or inhibitory effect on the modulation exerted by another modulatory system (Miles and Sillar, 2011). For example, glial-derived adenosine exerts its actions on the locomotor CPG through the modulation of DA signalling within the mouse spinal cord. Adenosine, acting via A1 receptors, inhibits the intracellular pathway downstream of D1 receptor activation, thus affecting dopaminergic modulation during drug-induced locomotion (Acevedo *et al.*, 2016; Acton and Miles, 2017). Another example of metamodulation is present in the swimming networks of *Xenopus larvae* tadpoles where nitric oxide modulates the release of NA in the spinal cord (McLean and Sillar, 2004). These examples demonstrate that metamodulation is an important mechanism in the regulation of the function of spinal motor networks (Miles and Sillar, 2011).

The experiments described in this thesis aimed to increase our understanding of one of the major intrinsic modulators of the mammalian locomotor CPG, ACh, for which many questions remain regarding its exact sources, roles and mechanisms of modulation. The sections below will address current knowledge of cholinergic modulation of spinal circuits.

1.4. Cholinergic modulation of locomotor circuits

Early studies highlighted ACh and its analogues as being responsible for the generation of muscle contraction (Bacq and Brown, 1936, 1937; Mcdowall and Watson, 1951). Groups of large cholinergic neurons, later referred to as MN pools (Romanes, 1951), were identified in the ventral horn of the spinal cord and it was shown that these cholinergic neurons innervated specific muscles according to their position in the periphery (Hollyday *et al.*, 1977; Landmesser, 1978).

The role of ACh within spinal locomotor networks initially attracted less attention. The concentrations of ACh and activity of ACh-esterase were shown to be lower in the spinal cord compared to the neuromuscular junction (Eccles *et al.*, 1956; Mitchell and Phillis, 1962). Early studies showed that exogenous ACh has both excitatory and inhibitory actions during ventral or dorsal root stimulation, with the opposing roles hypothesised to depend upon ACh concentration and the state of activity of the spinal cord (Eccles, 1947; Eccles *et al.*, 1956; Fernandez-de-Molina *et al.*, 1957; Mitchell and Phillis, 1962). Injection of ACh into the spinal cord was first shown to increase the electrical activity recorded from cervical rootlets in the cat (Feldberg *et al.*, 1953). Renshaw cells, a major population of glycinergic INs located close to MN pools, were first found to be activated through antidromic stimulation of motor nerves and were thought to account for the early descriptions of the effects of ACh and ACh-esterase inhibitors on the spinal cord (Eccles *et al.*, 1954, 1956). Ventral root discharges in response to ACh and depressant actions on spinal reflexes were later found on the cat lumbrosacral cord (Fernandez-de-Molina *et al.*, 1957). These early descriptions of ACh were the first evidence of cholinergic neuromodulatory effects in the spinal cord.

ACh can act on both ionotropic nicotinic (nAChR) and metabotropic muscarinic (mAChR) receptors. Early studies of the distribution of ACh receptors in the spinal cord suggested greater expression of mAChRs compared to nAChRs, with the former being mostly present in the ventral horn whereas ionotropic nAChRs are generally localized in the dorsal horn (Oguz Kayaalp and Neff, 1980; Gillberg *et al.*, 1988). Besides having different patterns of expression throughout the spinal cord, both nAChRs and mAChRs are involved in the control of both sensory and motor outputs within spinal circuits.

1.4.1. Spinal sources of acetylcholine

Most of the knowledge on the ACh receptors derives from insights obtained from functional approaches with very limited evidence available on the precise receptor distribution in the spinal cord. A slowing step has been the poor description of the different populations of cholinergic INs in the spinal cord. There are different, discrete groups of cholinergic INs both in the dorsal and ventral horns of the spinal cord. The cholinergic INs located dorsally are found in laminae II-IV and are involved in nociception and inhibition of cutaneous afferent input by modulating GABA and glycine release. These cholinergic dorsal INs do not seem to affect locomotor circuitry involved in the generation of rhythmic motor patterns (Todd, 1991; Kurihara *et al.*, 1993; Bordey *et al.*, 1996; Garraway and Hochman, 2001; Olave *et al.*, 2002; Stewart and Maxwell, 2003; Genzen and McGehee, 2005; Seddik *et al.*, 2006, 2007; Wang *et al.*, 2006; Takeda *et al.*, 2007; H. M. Zhang, Zhou, *et al.*, 2007; H. Zhang *et al.*, 2007; Cai *et al.*, 2009; Shelukhina *et al.*, 2009; Zhang *et al.*, 2009; Mesnage *et al.*, 2011; Liu *et al.*, 2011; Pawlowski *et al.*, 2013; Jeong *et al.*, 2013; Chen *et al.*, 2014). The remaining non-dorsal INs that release ACh in lumbar sections of the mammalian spinal

cord seem to be restricted to laminas VII and X (Gillberg *et al.*, 1988, 1990; Sherriff and Henderson, 1994; Bertrand and Cazalets, 2011). These include a group of bilaterally projecting cholinergic INs known as partition cells present in lamina VII close to the central canal (Sherriff and Henderson, 1994; Bertrand and Cazalets, 2011), $Pitx2^{+}$ INs (Miles *et al.*, 2007; Zagoraiou *et al.*, 2009), GABAergic and cholinergic cluster cells (Gotts *et al.*, 2016), and other unidentified groups of cholinergic INs scattered around this area (Oguz Kayaalp and Neff, 1980; Gillberg *et al.*, 1988, 1990; DenbSchäfera and Eidenb, 1998; Kobayashi *et al.*, 2002; Carlin, 2005; Liu *et al.*, 2009; Bertrand and Cazalets, 2011). From all these studies, $Pitx2^{+}$ INs remain the only group of cholinergic INs that can be genetically traced thus providing a reliable tool to study muscarinic modulation of spinal locomotor networks. Using Cre-lox recombination it has been possible to visualize $Pitx2^{+}$ INs and perform anatomical and functional studies of this subpopulation (Zagoraiou *et al.*, 2009).

1.4.2. Nicotinic acetylcholine receptors

In the Central Nervous System nAChRs are made up of 5 different subunits that can be divided into two distinct classes, either α (α2-α9) or β subunits (β2-β4). The assembly of specific subunits structurally and functionally defines the type of nAChR and its ionic conductance. Binding studies have suggested the presence of different types of subunits of nAChRs in the spinal cord (Khan *et al.*, 2003). However, the heteromeric α4β2 nAChR that is permeable to both Na^{+} and K^{+}, and the homomeric α7-subunit containing receptor that is highly permeable to Ca^{2+}, are the two subtypes of nAChRs that have been functionally described in the spinal cord (Alvarez *et al.*, 1999; A. Bradaïa and Trouslard, 2002). Despite not having a pronounced role in the

cholinergic modulation of CPG networks involved in hindlimb locomotion (Jordan et al. 2014), nAChRs do seem to be important for the activation of Renshaw Cells (Alvarez *et al.*, 1999; Mentis *et al.*, 2005; Nishimaru *et al.*, 2005; Lamotte d'Incamps and Ascher, 2008; Shelukhina *et al.*, 2009; O'Donovan *et al.*, 2010), the regulation of GABA and glycine release in the dorsal horn and near the central canal (Ren and Greer, 2003; Seddik *et al.*, 2006; Gonzalez-Islas *et al.*, 2016), nociceptive signalling (Garraway and Hochman, 2001; Bradaïa *et al.*, 2005; Takeda *et al.*, 2007; Shelukhina *et al.*, 2009), viscerosensory transmission (Bordey *et al.*, 1996; A Bradaïa and Trouslard, 2002; Bradaïa *et al.*, 2005; Seddik *et al.*, 2006; Ogier *et al.*, 2008) and embryonic development of locomotor networks (Milner and Landmesser, 1999; Ren and Greer, 2003; Myers *et al.*, 2005).

In the dorsal horn, nAChRs are thought to be involved in the inhibition of nociceptive signalling via facilitation of inhibitory transmission to dorsal INs, mainly through α4β2 and α7-subunit containing nAChRs (Garraway and Hochman, 2001; Bradaïa *et al.*, 2005; Takeda *et al.*, 2007; Shelukhina *et al.*, 2009). Activation of nAChRs has also been implicated in the control of GABA and glycine release in the dorsal horn and near the central canal (A. Bradaïa and Trouslard, 2002; Ren and Greer, 2003; Bradaïa *et al.*, 2005; Seddik *et al.*, 2007; Gonzalez-Islas *et al.*, 2016). This cholinergic downregulation of inhibitory release during development helps to maintain sufficient spontaneous network activity in the embryonic network thus contributing to appropriate assembly of network connectivity and synaptic fidelity (Ren and Greer, 2003; Gonzalez-Islas *et al.*, 2016). The actions of nAChRs on GABAergic and glycinergic transmission are also involved in the modulation of sympathetic

preganglionic neurones (Seddik *et al.*, 2007) and lamina X INs (Bradaïa & Trouslard 2002; Bradaïa et al. 2005) in the neonatal rat.

The non-selective nAChR blocker, tubocurarine, has no effect on ACh-induced excitation of rat lumbar locomotor networks, achieved using ACh-esterase inhibitors (Jordan *et al.*, 2014), suggesting that nAChRs do not have a prominent role in the modulation of the spinal CPG. However, nAChRs are involved in the activation of Renshaw cells whose activation by MNs modulates inhibitory inputs back to MNs thus resulting in recurrent inhibition. MNs have axon collaterals that project back into the spinal cord and contact Renshaw cells. At these synapses MNs release glutamate and ACh with blockade of nAChRs containing α4β2 and α7-subunits reducing the effect of MN activation on Renshaw cells (Alvarez *et al.*, 1999; Mentis *et al.*, 2005; Nishimaru *et al.*, 2005; Lamotte d'Incamps and Ascher, 2008; O'Donovan *et al.*, 2010). Despite the lack of evidence of involvement of nAChRs in the generation of locomotor activity in the newborn rat (Jordan *et al.*, 2014), in early embryonic stages nAChRs seem to be responsible for bursts of patterned locomotor-like activity (Milner and Landmesser, 1999; Hanson and Landmesser, 2003; Myers *et al.*, 2005) which are important for appropriate assembly of spinal locomotor circuitry during development (Myers *et al.*, 2005).

1.4.3. Muscarinic acetylcholine receptors

There are 5 types of mAChRs (M1-M5) found in the central nervous system (Scarr, 2012). Within the spinal cord, only M2 and M3 muscarinic receptors appear to play an active role in the modulation of sensory and motor networks (Kurihara *et al.*, 1993; Jordan *et al.*, 2014). M2 muscarinic receptors act via G_i proteins which decrease

the levels of cAMP generally leading to inhibitory effects, whereas M3 receptors are coupled to the G_q class of proteins which activate phospholipase C and up-regulate the intracellular levels of Ca^{2+} resulting in excitation. In addition to their canonical pathways, in the central nervous system mAChRs can activate multiple signalling cascades that depend on the type of synapse being studied making it difficult to predict the physiological consequences of the activation of a certain subtype of receptor (Scarr, 2012). Therefore, throughout this work, the role of both M2 and M3 muscarinic receptors will be detailed and explored based on physiological evidence from previous works and the data obtained.

Application of muscarine to isolated segments of the cat spinal cord provided early evidence of the presence of excitatory muscarinic receptors on lateral horn cells (Yoshimura and Nishi, 1982). In later studies, muscarine was shown to depolarize rat MNs (Jiang and Dun, 1986) and also brainstem astrocytes (Hösli *et al.*, 1988). Early immunohistochemical and binding studies indicated the presence of mAChRs in the spinal cord (Oguz Kayaalp and Neff, 1980; Gillberg *et al.*, 1988). The density of mAChRs is greater in the ventral horn compared to the dorsal horn (Oguz Kayaalp and Neff, 1980), which could suggest that these receptors have a more prominent role in the modulation of CPG networks involved in the generation of rhythmic motor patterns. The precise location of M2 and M3 muscarinic receptors in the spinal cord has not been completely characterized. Autoradiographic studies have indicated the presence of M2 receptors in both ventral and dorsal horns whereas M3 receptors were mostly present in the dorsal horn (Höglund and Baghdoyan, 1997). In adult rats, large MNs (diameters greater than 35μm) displayed intense immunorreactivity across the plasma membrane for the M2 receptor whereas small MNs were weakly labelled (Welton *et al.*, 1999).

Large MN somas are usually attributed to alpha MNs whereas small MN cell bodies are presumably gamma MNs, thus, M2 receptor expression might vary across different MN subtypes (Welton *et al.*, 1999). In another study it was shown that clusters of the M2 receptor on MNs were juxtaposed with large, cholinergic C-bouton terminals (Hellström *et al.*, 2003) that were later revealed to derive from Pitx2^{+} INs (Miles *et al.*, 2007; Zagoraiou *et al.*, 2009). The distribution of M3 muscarinic receptors on MNs is less understood with immunohistochemical studies suggesting that these receptors are present in fine cytoplasmic puncta on MNs (Wilson *et al.*, 2004).

1.4.3.1. Role of mAChRs in the modulation of spinal locomotor networks

The first evidence of a modulatory role for muscarinic receptors within spinal circuits came from studies performed in the neonatal rat in which ventral root potentials were recorded after electrical stimulation of the saphenous nerve in isolated spinal cord-saphenous nerve preparations or after application of noxious skin stimuli in isolated spinal cord-saphenous nerve-skin preparations (Kurihara *et al.*, 1993). This work demonstrated that activation of M2 muscarinic receptors decreased whereas activation of M3 receptors increased saphenous nerve-evoked potentials. M2 receptor activation also potentiated the ventral root potential evoked by capsaicin application to skin (Kurihara *et al.*, 1993). This highlighted a role for muscarinic modulation of spinal networks involved in nociceptive transmission. Due to the presence of mAChRs in the dorsal horn it would be expected that these receptors would play a role in the regulation of sensory inputs. In addition, likely due to the prevalence of mAChRs in the ventral

horn, a prominent role for mAChRs in the modulation of CPG networks was also later described.

To understand the modulatory role of cholinergic receptors in mammalian spinal locomotor circuits researchers have recorded ventral root output in isolated neonatal rat spinal cords while increasing the levels of ACh. Elevation of ACh levels in the spinal cord of the neonatal rat can be achieved by exogenous application of ACh or inhibition of the ACh-esterase enzyme (Cowley and Schmidt, 1994, 1997; Kiehn *et al.*, 1996; Jordan *et al.*, 2014). Although both methods have been shown to induce locomotor-like bursts of activity, the activity is erratic and does not exhibit appropriate alternation between left and right sides and antagonist muscles. Thus elevation of ACh probably leads to an increase in the excitability of the CPG network that does not contribute to adequate rhythm. The use of muscarinic antagonists revealed that these locomotor-like bursts are initiated by the activation of M3 muscarinic receptors that excite CPG neurons. On the other hand, M2 antagonism decreased the amplitude of ACh-induced activity, suggesting that this receptor subtype might be important in the modulation of the strength of the motor output. The use of nicotinic receptor blockers and other muscarinic receptor antagonists did not affect ACh-elicited bursts, revealing that M2 and M3 muscarinic receptors are solely responsible for cholinergic modulation of CPG networks in the rat (Jordan *et al.*, 2014). Muscarine has also been shown to evoke irregular bursts of activity in the neonatal mouse spinal cord (Jiang *et al.*, 1999) with M2 receptor blockade decreasing burst amplitude during NMDA, DA and 5-HT-induced locomotion (Miles *et al.*, 2007).

Groups of cholinergic INs in the sacral region of the spinal cord project rostrally to the lumbar segments and modulate the locomotor CPG through M2 muscarinic

receptor activation. Electrical stimulation of sacral afferents in the neonatal rat can evoke rhythmic bursts of activity that can be recorded through suction electrodes attached to lumbar ventral roots (Etlin *et al.*, 2014; Finkel *et al.*, 2014; Anglister *et al.*, 2017). Restricted application of ACh-esterase inhibitors to the sacral region of the spinal cord during stimulation increases burst amplitude and slows the frequency of the lumbar rhythm. Both of these effects are blocked by M2 muscarinic receptor antagonism. Anatomical studies have shown that clusters of cholinergic neurons provide ascending excitatory projections from the sacral segments of the spinal cord into the lumbar region through the ventral funiculus and the lateral white matter funiculi. These groups of rostral projecting cholinergic sacral neurons are thought to mediate the described physiological network effect of ACh (Etlin *et al.*, 2014; Finkel *et al.*, 2014; Anglister *et al.*, 2017).

The limited studies of the role of M2 and M3 muscarinic receptors in mammalian locomotor circuits suggests that these receptors differently modulate rhythmic output. M3 muscarinic receptors are important in the cholinergic activation of neurons within the locomotor network that might drive the CPG (Jordan *et al.*, 2014) whereas M2 receptors seem to regulate the amplitude of the motor output (Miles *et al.*, 2007; Jordan *et al.*, 2014; Anglister *et al.*, 2017). These actions of M2 and M3 receptors on locomotor output might reflect the effects of ACh on the cellular properties of different spinal INs and MNs that comprise the mammalian locomotor circuits.

1.4.3.2. Physiological effects of mAChRs on spinal INs and MNs

The effects of muscarinic receptor activation have been studied in both dorsal and ventral INs as well as in MNs. These effects are likely to account for the previously

described actions of muscarinic receptors on sensory and motor networks. In spinal sensory circuits, M2 and M3 muscarinic receptors are involved in afferent fibre-evoked nociception in the neonatal rat spinal cord (Kurihara *et al.*, 1993). In the dorsal horn M3 receptors potentiate glycinergic release whereas the M2 subtype enhances the release of GABA and reduces the release of glutamate to INs which are important for spinal nociception (Wang *et al.*, 2006; Cai *et al.*, 2009; Zhang *et al.*, 2009; Jeong *et al.*, 2013; Chen *et al.*, 2014).

A range of different effects of muscarinic receptor activation has been observed in ventral horn INs that are part of the mammalian CPG. In the neonatal rat spinal cord, application of muscarine depolarizes INs located around the central canal, increases the amplitude of voltage dependent burst oscillations and leads to a more unstable locomotor rhythm when coapplied with NMDA and 5-HT (Kiehn *et al.*, 1996). Experiments using organotypic spinal cord slices from rat embryos have demonstrated that exogenous application of muscarine increases intrinsic spiking in the ventral horn area by depolarizing and triggering firing in silent spinal neurons (Czarnecki *et al.*, 2009). In mice expressing the enhanced green fluorescent protein (EGFP) driven by the c-fos promoter it is possible to visualize active INs after perfusion of locomotor drugs or behavioural tasks. In $EGFP^+$ INs from these animals, ACh increased firing output, decreased action potential threshold, increased input resistance, decreased mAHP amplitude and decreased the hyperpolarization activated current (I_h) (Dai *et al.*, 2009; Dai and Jordan, 2010). Given their role in whole locomotor network output (Jordan *et al.*, 2014), it would be expected that these observations reflect modulation of M2 and M3 muscarinic receptors in INs. However, this remains to be determined because M2 and M3 receptor antagonists were not employed in these previous studies.

Studies focused on the characterization of cholinergic modulation of MNs have revealed a variety mAChR-dependent mechanisms affecting MN excitability. In the salamander spinal cord, muscarine increases MN firing output, decreases the mAHP, decreases the hyperpolarization activated current (I_h), positively modulates I_{KIR} but has no effect on spike amplitude, width or input resistance (Chevallier *et al.*, 2006, 2008). Recordings from MNs from neonatal mouse spinal cord slices have also demonstrated a muscarine-induced increase in MN firing output that is dependent on M2 receptor activation and appears to result from a decrease in the mAHP amplitude which leads to an increase in the number of evoked spikes (Miles *et al.*, 2007). A group of bilaterally projecting cholinergic INs in lamina VII close to the central canal, known as partition cells, (Sherriff and Henderson, 1994), and commissural cholinergic INs located in lamina X (Bertrand and Cazalets, 2011) are known to project to contralateral MNs. These cholinergic INs are thought to enhance locomotor output by causing the closure of the slowly activating voltage-regulated K^+ current (M-current), which is dependent on muscarinic receptor activation (Bertrand and Cazalets, 2011). M3 muscarinic receptors present at the synapses between these commissural cholinergic terminals and MNs were suggested to be involved in this modulation which is also able to depolarize MNs (Bertrand and Cazalets, 2011). The synaptic drive to MNs mediated by mAChRs has been shown to originate from ventromedial INs near the central canal (which includes $Pitx2^+$ INs), thus suggesting that muscarinic modulation of locomotor output might be anatomically restricted to this area in the spinal cord (Gillberg *et al.*, 1988, 1990; Sherriff and Henderson, 1994; Bertrand and Cazalets, 2011).

Cholinergic-dependant changes in the output of INs involved in rhythm stability (Kiehn *et al.*, 1996; Dai *et al.*, 2009; Dai and Jordan, 2010) and MNs (Chevallier *et al.*,

2006, 2008; Miles *et al.*, 2007) indicate that ACh modulates cellular properties of neurons that shape both the rhythm as well and the strength of motor bursting. The cholinergic modulation of CPG INs and MNs will shape the network output recorded from ventral root bursting (Jordan *et al.*, 2014).

Despite the above-mentioned studies, the cellular mechanisms and the exact role of M2 and M3 muscarinic receptor-mediated modulation of the mammalian locomotor CPG remains to be fully addressed. Although studies have addressed M2 and M3 receptor-mediated modulation of ACh-induced bursts of activity (Cowley and Schmidt, 1994, 1997; Kiehn *et al.*, 1996; Jordan *et al.*, 2014), this output lacks the physiological pattern of locomotor-like rhythm which is observed during NMDA, DA and 5-HT-induced bursting (Jiang *et al.*, 1999). The majority of the studies performed on INs and MNs only assessed the pharmacological effects of ACh and the non-selective muscarinic receptor antagonist oxotremorine (Dai *et al.*, 2009; Dai and Jordan, 2010), which prevents any conclusions from being drawn regarding the receptor subtypes involved. The work described in this thesis attempts to provide valuable information regarding the physiological effects of M2 and M3 muscarinic receptors on MN function and understand the impact of this muscarinic modulation in appropriate alternating and rhythmic lumbar locomotor activity. Another impediment towards a more complete understanding of muscarinic actions on spinal motor circuits is the lack of well-defined genetic markers for cholinergic neurons in the spinal cord. Taking advantage of the Cre-lox genome engineering technology, the work in this thesis also focused on understanding the role of genetically traceable cholinergic, $Pitx2^{+}$ INs in the control of locomotor networks.

1.5. Pitx2^{+} spinal cholinergic INs

Cholinergic, Pitx2^{+} INs of the spinal cord are known to form specialised synaptic contacts with MNs, termed C-boutons. C-boutons were first described in early studies characterizing the structure of different synaptic contacts on cat MNs. In these early studies large C-shaped presynaptic terminals that synapsed with MNs were termed C-boutons (Conradi, 1969; Conradi and Skoglund, 1969). C-boutons were later reported in different animals including humans (Pullen *et al.*, 1992) and mice (Wilson *et al.*, 2004). The cellular origin of C-bouton synapses was unknown when they were first described. Later work provided evidence that C-boutons expressed cholinergic markers (Li *et al.*, 1995). It was eventually found that C-boutons derive from cholinergic INs clustered around the central canal, which are marked by expression of the transcription factor Pitx2 (Miles *et al.*, 2007; Zagoraiou *et al.*, 2009).

Several proteins have been identified at C-bouton synapses. The M2 muscarinic receptor is located postsynaptically along with the voltage-gated K^{+} channel 2.1 (Kv2.1), N-type Ca^{2+} channels and the SK channel (Wilson *et al.*, 2004; Deardorff *et al.*, 2014). Postsynaptic sigma-1 receptors have also been identified at the C-bouton synapse beneath the plasma membrane, on subsurface cisternae (Mavlyutov *et al.*, 2012). Immunolabelling studies have suggested the presence of nAChRs (Khan *et al.*, 2003), urotensin II receptors (Bruzzone *et al.*, 2010) and ATP receptors (Deng and Fyffe, 2004) at the presynaptic site.

Genetic tools have facilitated detailed studies of the anatomy and function of C-boutons in spinal networks. Although cholinergic C-boutons derive from Pitx2^{+} INs, this population of INs can be subdivided into two different phenotypes: cholinergic Pitx2^{+} neurons that express choline acetyltransferase (ChAT) and vesicular

acetylcholine transporter (vAChT), and glutamatergic Pitx2^{+} neurons that express vesicular glutamate transporter 2 (vGlutT2). At the upper lumbar level Pitx2^{+} INs are predominantly cholinergic. The majority of Pitx2^{+} INs that express vGlutT2 are found in more caudal segments. In the lumbar cord, cholinergic Pitx2^{+} INs are outnumbered by MNs by a factor of 1:10 with each individual Pitx2^{+} IN forming up to 1000 synapses with MNs resulting in approximately 80-100 C-bouton contacts per MN. This high innervation rate is suggestive of a general modulatory role in MN function. The majority of Pitx2^{+} INs project ipsilaterally, while one third also branch contralaterally. Cholinergic Pitx2^{+} INs also appear to project to spinal INs around the ventromedial area, however the specificity of this connection is unclear (Zagoraiou *et al.*, 2009). It has also been suggested that the distribution of modulatory inputs from cholinergic cells around the central canal (which includes Pitx2^{+} INs) might be more complex. Some of these cholinergic INs project ipsilaterally whereas others bifurcate bilaterally through several segments of the lumbar spinal cord (Stepien *et al.*, 2010). Recent work has shown that terminals from neurons of the lateral vestibular nucleus involved in co-activation of extensor and flexor muscles, that are important for postural control, were found near cholinergic Pitx2^{+} INs. Authors suggested that perhaps these INs might be part of a pathway involving MN modulation through Pitx2^{+} INs, resulting in muscle co-activation (Murray *et al.*, 2018). In fact, bilateral projecting cholinergic INs have been observed to project to MN pools responsible for the control of posture (Stepien *et al.*, 2010).

Given the complex anatomy of Pitx2^{+} INs, functional readouts have provided much of the insights into the physiological effects of these INs on locomotor output. Most of the Pitx2^{+} INs are tonically active at rest and receive glutamatergic,

GABAergic and serotonergic inputs that might drive Pitx2$^+$ INs activity (Zagoraiou *et al.*, 2009). These inputs are not derived from premotor spinal INs (Stepien *et al.*, 2010), suggesting that this subpopulation receives connections from different descending and/or local projections that could trigger activation of Pitx2$^+$ INs. In fact, their spiking increases during fictive locomotion (NMDA, DA and 5-HT-induced) with firing patterns that are in phase with segmentally-aligned ventral root bursts (Zagoraiou *et al.*, 2009). Since cholinergic Pitx2$^+$ INs are part of the locomotor network, provide modulatory inputs to MNs and are active during locomotion, behaviour experiments were carried out to understand the role of this population in adult animals (Zagoraiou *et al.*, 2009). The expression of ChAT was genetically supressed at the C-bouton synapse in mice, thus rendering cholinergic Pitx2$^+$ INs modulation inefficient. Authors then recorded gastrocnemius activation through electromyograms during walking followed by swimming (Zagoraiou *et al.*, 2009). The activation of hindlimb muscles is greater during swimming when compared to walking (Leon *et al.*, 1994), and elimination of cholinergic modulation from C-boutons enable a understanding of the contribution of cholinergic Pitx2$^+$ INs in different degrees of muscle activation. Analysis during swimming tests revealed that these animals exhibited decreased hindlimb muscle activity when compared to control mice. This suggested that cholinergic Pitx2$^+$ INs might exhibit a task-dependent modulation by enhancing MN firing when higher muscle activation is required (Zagoraiou *et al.*, 2009).

The exact mechanism through which Pitx2$^+$ INs modulate MN function is still not fully defined. Indirect evidence suggests that activation of Pitx2$^+$ INs and therefore C-boutons regulates motor output through M2 muscarinic receptors to match muscle activation with behavioural demands (Miles *et al.*, 2007; Zagoraiou *et al.*, 2009). This

could be achieved through M2 receptor-mediated inhibition of SK channels which would reduce the mAHP thus allowing for increased MN firing rate (Miles *et al.*, 2007). However, other channels which are present in MNs soma juxtaposed to C-boutons, such as Kv2.1 and N-type Ca^{2+} channels, could also be involved in M2 receptor-mediated regulation of MN output (Witts *et al.*, 2014).

This subpopulation of INs also seems to play a role in dysfunction and disease. Cholinergic inputs to motoneurons are markedly reduced after spinal cord injury (Kapitza *et al.*, 2012; Skup *et al.*, 2012) and sprouting of cholinergic fibres at the injury site is known to be important for recovery of hindlimb stepping (Jakeman *et al.*, 1998). This decreased cholinergic innervation after spinal cord injury has functional implications for locomotor behaviour. Reduced numbers of these C-boutons onto MNs in spinal cord injury have been implicated in the rapid exhaustion of neuronal activity underlying locomotor output (Kapitza *et al.*, 2012) and previous experiments in spinalized cats have shown a parallel between the increase in the number and size of these C-boutons and recovery of locomotor function (Pullen and Sears, 1983). Changes in cholinergic connectivity in the spinal cord is also accompanied by a hyper-cholinergic state involving spinal muscarinic receptor function (Jordan *et al.*, 2014). In neurodegenerative diseases, such as Amyotrophic Lateral Sclerosis (ALS) abundance and enlargement of C-boutons relates to disease pathophysiology (Herron and Miles, 2012; Milan *et al.*, 2015). When studying the development of $Pitx2^{+}$ IN contacts with MNs in mice, authors found that in healthy animals there is a maturation process involving an increase in the area of the C-bouton synapse and increased expression and clustering of postsynaptic M2 muscarinic receptors. In the SOD1(G93A) mouse model of ALS C-boutons are smaller at birth and their density increases throughout early post-

natal development with a decline around symptomatic stages that is followed by decreased M2 muscarinic receptor expression in the spinal cord (Milan *et al.*, 2015). This will ultimately lead to reduced MN modulation by cholinergic Pitx2^{+} INs in ALS and therefore decreased muscle activation. Recent evidence suggests that Pitx2^{+} INs express the α3-subunit of the Na^{+}/ K^{+} ATPase, which is thought to be responsible for an ultra-slow AHP that is important for activity-dependent changes in motor function. Mutations in the α3-subunit are related with diseases impairing the control of movement such as alternating hemiplegia of childhood and rapid-onset dystonia Parkinsonism and CAPOS syndrome (Picton *et al.*, 2017).

Given the range of pathological conditions that may involve Pitx2^{+} INs and C-boutons, a greater understanding of the mechanisms by which Pitx2^{+} INs modulate motor output will not only provide valuable information about the physiology of spinal circuits involved in the control of movement, but also important knowledge which may help to develop therapeutic approaches to treat spinal injury and disease.

2. RESEARCH FRAMEWORK

The knowledge of the circuits and signalling pathways responsible for the generation of rhythmic motor patterns in the mammalian nervous system is incomplete. Despite the identification of several populations of spinal INs that express specific transcription factors allowing concise anatomical and behavioural studies, there is still knowledge lacking regarding the modulation of the mammalian CPG (Goulding, 2009; Miles and Sillar, 2011; Arber, 2012).

ACh is an important intrinsic neuromodulator arising from different INs from within the spinal cord, that is able to adjust the rhythmicity of the CPG and fine-tune the strength of motor output. Muscarinic receptors are thought to be accountable for the cholinergic actions on spinal networks responsible for the generation of movement with M2 and M3 muscarinic receptors being the subtypes involved (Miles *et al.*, 2007; Bertrand and Cazalets, 2011; Jordan *et al.*, 2014; Anglister *et al.*, 2017). However, previous studies have only partially addressed the roles and mechanisms of these receptors on CPG networks. The first part of this thesis therefore aimed to (1) describe the physiological relevance of these receptors for spinal motor circuits controlling locomotion and (2) explore and define the cellular mechanisms underlying M2 and M3 muscarinic receptor modulation of MN function.

After characterizing the role of M2 and M3 receptors in the modulation of locomotor network output and MN function, the next aim of this thesis was to understand which particular subset of cholinergic INs underlies some of the muscarinic modulation of spinal locomotor control networks. Pitx2^{+} INs are the only subpopulation of cholinergic INs that can be genetically identified and are thought to modulate motor output through M2 muscarinic receptors (Zagoraiou *et al.*, 2009; Witts *et al.*, 2014).

Prior to the current study, no research had addressed the direct effect of Pitx2^{+} IN activation on motor output. Cre-lox recombination provides a resourceful instrument to study the role of these neurons by allowing the selective expression of proteins in Pitx2^{+} INs such as fluorescent tags or Designer Receptor Exclusively Activated by Designer Drugs (DREADDs). DREADD technology allows the expression of excitatory (CHRM3) or inhibitory (CHRM4) engineered receptors in neurons that can only be activated with a designer drug (Roth, 2016; Zhu *et al.*, 2017). Expression of DREADDs in Pitx2^{+} INs combined with the use of muscarinic receptor antagonists was used to: (1) excite Pitx2^{+} INs and investigate the fundamental mechanism underlying changes in MN excitability and (2) inhibit Pitx2^{+} INs during fictive locomotion and study their role in the modulation of rhythmic bursts of locomotor-related activity.

Genetic deletion of neuronal populations has been successfully utilised to determine the role of different subtypes of INs within spinal circuits (Goulding, 2009; Arber, 2012). Therefore, after detailing the mechanisms of Pitx2^{+} INs-mediated modulation of motor output, cre-lox recombination was finally used to genetically ablate Pitx2^{+} cells to determine the effects on rhythmic locomotor network output. Using muscarinic receptor pharmacology in these animals during fictive locomotion, the physiological roles of the Pitx2^{+} IN system were further revealed.

3. METHODS

3.1. Animal ethics and husbandry

All the procedures described in this work were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 under Home Office project licences (PPL 6004369 and P6F7B721E) held by Dr Gareth B. Miles and personal licence (I27F5EFDC) held by Filipe Nascimento. Adult mice were crossed in pairs or trios (2 females and 1 male) for no longer than 6 litters in double or 12 litters in triple housed cages. Animals younger than 12 months were used for breeding.

3.2. Genotyping

For genetically modified mice (GM mice) a polymerase chain reaction (PCR) was conducted using DNA isolated from neonatal tail or adult ear samples. A tissue dissociation kit (Sigma Aldrich®) was used to obtain genomic DNA that was then added to the REDExtract-N-Amp PCR Reaction Mix (Sigma Aldrich®) along with required primers for DNA amplification using a standard PCR reaction (with 35-50 cycles). The amplified DNA was then directly loaded to an agarose gel (1.5%) and a gel electrophoresis was used to separate the fragments based on their molecular weight. DNA bands were visualized under ultraviolet light.

3.3. Animal lines

C57/BL6 neonates from postnatal day 1-12 were used for standard experiments. These animals do not carry any mutation that affects motor phenotype and thus are considered as wild type (WT). To visualize $Pitx2^{+}$ INs cre-lox recombination was used by crossing *Pitx2::Cre* (W. Liu *et al.*, 2003) with homozygous *ROSA-loxP-STOP-loxP-*

tdTomato fluorescent reporter animals (Madisen *et al.*, 2010). The *Pitx2::Cre:ROSA-loxP-STOP-loxP-tdTomato* mouse (Pitx2-Cre;TdTomato) exhibits fluorescence allowing Pitx2$^+$ INs to be targeted for single cell electrophysiology (Zagoraiou *et al.*, 2009). *Pitx2::Cre* and *Pitx2::Cre:ROSA-loxP-STOP-loxP-tdTomato* mice were crossed with *B6N;129-Gt(ROSA)26Sortm2(CAG-CHRM3*,-mCitrine)Ute/J* animals in order to generate Pitx2-Cre;CHRM3 and Pitx2-Cre;TdTomato;CHRM3 offspring which express the hM3Dq DREADD receptor in Pitx2$^+$ INs. Pitx2$^+$ mice were also crossed with *B6N.129-Gt(ROSA)26Sortm1(CAG-CHRM4*,-mCitrine)Ute/J* to obtain Pitx2-Cre;CHRM4 and Pitx2-Cre;TdTomato;CHRM4 animals that express the hM4Di DREADD receptor in Pitx2$^+$ INs. The chemical activation of these engineered signalling proteins can be triggered by CNO which will increase or induce Pitx2$^+$ IN firing (hM3Dq through G_q pathway) or reduce their activity (hM4Di through G_i signalling) (Zhu *et al.*, 2017). Crossing *Pitx2::Cre* with *loxP-STOP-loxP-DTA* (made by L. Zagoraiou, Academy of Athens) animals (producing Pitx2-Cre;DTA offspring) allowed the expression of diphtheria toxin A in cholinergic Pitx2$^+$ INs thus selectively ablating this particular subset of cells.

3.4. *In vitro* spinal cord preparation isolation

Spinal cords from neonatal mice were obtained as previously described (Jiang *et al.*, 1999). Neonatal mice (postnatal day 0-12) were euthanized using cervical dislocation (schedule 1 killing procedure), decapitated and eviscerated. The animal was pinned into a chamber containing "dissecting" artificial cerebrospinal fluid (aCSF) continuously gassed with 95% O_2 and 5% CO_2 at a temperature of ~4°C. Spinal

vertebrae were cut and whole spinal cord was isolated from cervical to upper sacral segments.

For spinal cord slice preparation, both the ventral and dorsal roots were trimmed and the cord was glued to a 3% agar support. This was placed in a vibrating microtome apparatus (Leica VT1200) to obtain 300µm transverse slices from the lumbar segments. Slices were then transferred to a "recovery" aCSF solution continuously gassed with 95% O_2 and 5% CO_2 and at ~34ºC for 45-60min, before being transferred to a beaker with "recording" aCSF gassed with 95% O_2 and 5% CO_2 at room temperature (figure 3.4a). For ventral root recordings, dorsal roots were trimmed and ventral roots from L1-L5 were kept intact. For patch clamp recordings in intact, whole spinal cords, small vertical cuts were performed in the ventral meninges on the surface of the preparation next to the ventral root projection, allowing access to MN pools (figure 3.4b).

3.4.1. Whole-cell patch-clamp recordings from spinal neurons

Whole-cell patch-clamp recordings were made from spinal MNs, which were identified based on their size and location. INs are distributed throughout the ventral horn, while the larger MNs are organized in discrete pools in the ventromedial and ventrolateral spinal cord. Once recordings were established, MN identity was further verified based upon their larger whole-cell capacitance (C_m), lower input resistance, and greater synaptic drive compared to spinal INs (Carlin *et al.*, 2000; Witts *et al.*, 2015; Picton *et al.*, 2017). Identification and recordings of $Pitx2^+$ INs, which reside close to the central canal, were aided by their expression of fluorescent proteins (Zagoraiou *et al.*, 2009).

Whole spinal cord preparations or spinal cord slices were immersed in a recording chamber with recording aCSF continuously re-perfused (50mL) at a constant rate (approximately 1 mL per second). Borosilicated glass microelectrodes with resistance between 2.5-6MΩ were pulled and filled with an intracellular solution and attached to an Ag-AgCl recording electrode. The reference electrode was an Ag-AgCl pellet immersed in the bath. Signals were amplified and filtered (4-kHz low-pass Bessel filter) with a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA) and acquired at ≥10 kHz using a Digidata 1440A A/D board and pClamp software (version 10.6, Molecular Devices, Sunnyvale, CA, USA). A gigaohm seal (≥2GΩ) was obtained using negative pressure in the patch pipette, prior to the establishment of whole-cell mode in which the neuron was typically held at -60mV when in voltage-clamp mode. Acquisition of spontaneous postsynaptic currents (PSCs), miniature postsynaptic currents (mPSCs), drug-induced currents and measurements of input resistance (by applying voltage steps; 2.5mV steps from -75 to -52.5mV) were performed in voltage-clamp mode (V_{hold} at -60mV) (figure 3.4c). Neurons with an holding current between 100 and -100pA for control were selected for experiments and outwards currents were considered as increases in positive current whereas inward currents were defined as an increase in negative current. Firing output was measured in current-clamp mode either using 'gap-free' acquisition for spontaneous firing, by applying 10ms supramaximal pulses to evoke single action potentials, by injecting 1s square current pulses (starting at 10pA with 50pA increases), or through a single supramaximal ramp pulse (1s long). For adequate comparisons, a bias current was applied in all current-clamp protocols to keep neurons at the same resting potential

(figure 3.4d). There was no correction for the liquid junction potential, which was calculated as 14.2mV for the solutions used (Clampex JPCalcW).

3.4.2. Ventral root recordings

Whole spinal cords were pinned or glued ventral-side up in a recording chamber continuously perfused with recording aCSF and glass suction electrodes were attached to lumbar ventral roots (L1-L5). Locomotor-related activity (fictive locomotion) was triggered by bath perfusion of NMDA (5µM), 5-HT (10µM) and DA (50µM). Using high concentrations of these drugs contributes to the chemical excitation of CPG neurons in the spinal cord that will result in well-defined bursts of activity from the ventral roots that resemble the pattern of walking. Locomotor-related output is characterized by rhythmic bursts of activity which alternate between the right and left sides of the spinal cord and between ipsilateral extensor (L5) and flexor (L2) ventral roots (Jiang *et al.*, 1999). Rhythmic bursting was considered suitable for experiments when the frequency, duration and amplitude was stable for at least 15-20min. Signals were filtered and amplified (band-pass filter 30–3000 Hz, Qjin Design) and then acquired at a frequency of 6kHz with a Digidata 1440A A/D board and pClamp software. Custom built amplifiers (Qjin design) allowed acquisition of raw signals with simultaneous online rectification and integration (50-ms time constant).

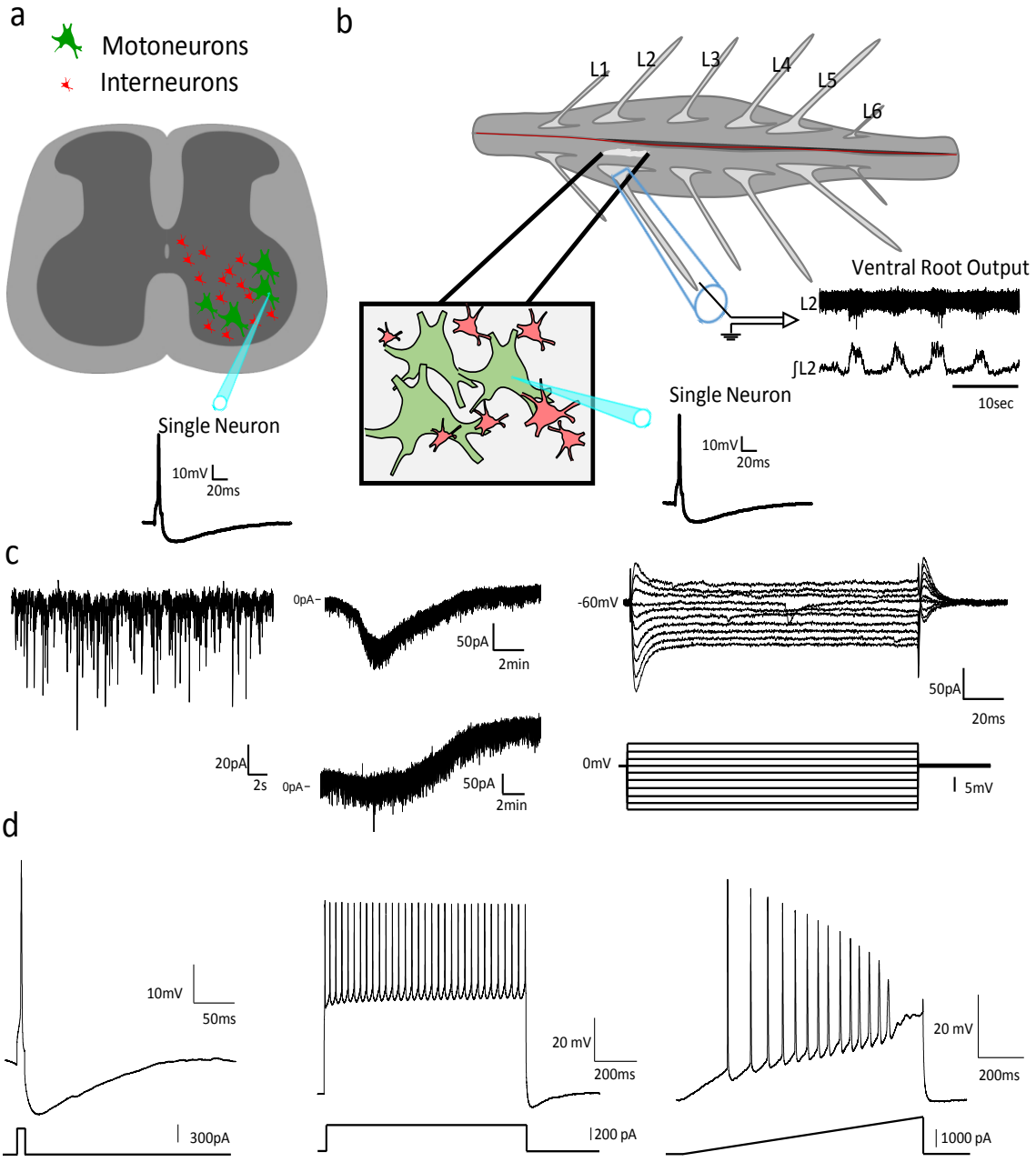


Figure 3.4 – Representation of a spinal cord slice and whole cord preparation with the different patch-clamp protocols used. **(a)** schematic illustrating the position of MNs and INs throughout the surface of a spinal cord slice; **(b)** lumbar segments of the whole spinal cord with suction electrode attached to the L2 lumbar root and an example of recorded locomotor-related output (raw trace on top and integrated/rectified on bottom). Inset shows MNs in a motor pool from the same segment with representation of patch electrode recording; **(c)** voltage-clamp protocols: PSCs recorded from a MN in gap-free mode (left), examples of an inward (middle, top) and outward (middle, bottom) currents elicited in a MN via drug application and voltage steps applied to a MN to evaluate changes in input resistance (right); **(d)** current clamp protocols: 10ms-duration current pulse injected to evoke a single action potential (left), example of 1s-duration current pulse used to elicit repetitive firing (middle) and a MN response to a 1s-duration current RAMP (right).

3.5. Drugs

The dissecting aCSF contained (in mM): 25 NaCl, 188 sucrose, 1.9 KCl, 1.2 NaH_2PO_4, 10 $MgSO_4$, 1 $CaCl_2$, 26 $NaHCO_3$, 25 glucose and 1.5 kynurenic acid. The recovery solution contained (in mM): 119 NaCl, 1.9 KCl, 1.2 NaH_2PO_4, 10 $MgSO_4$, 1 $CaCl_2$, 26 $NaHCO_3$, 20 glucose and 1.5 kynurenic acid. The recording aCSF contained (in mM): 127 NaCl, 3 KCl, 1.25 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$, 26 $NaHCO_3$, 10 glucose. The intracellular solution for patch-clamp recordings was made of (in mM): 14 $KMeSO_4$, 10 NaCl, 1 $CaCl_2$, 10 HEPES, 1 EGTA, 3 Mg-ATP and 0.4 GTP-Na_2 (pH 7.2–7.3, adjusted with KOH). Muscarine, 4-DAMP, methoctramine, NMDA, DA and 5-HT were supplied by Sigma-Aldrich®; tetrodotoxin (TTX) by Bio-Techne®; CNO by Tocris® and Hello-Bio®; and guangxitoxin-1E by Alomone Labs®. All drugs were dissolved in H_2O except for 4-DAMP which was dissolved in DMSO to a concentration that did not exceed 0.1% (vol/vol) in working solutions. Drug application was performed for 20-30 minutes except for CNO in which perfusion lasted 15 minutes to avoid desensitization and subsequent DREADD receptor downregulation (Roth, 2016).

3.6. Data analysis

Whole-cell patch-clamp data were analysed with Clampfit (Molecular Devices, Sunnyvale, CA, USA) and, for PSCs and mPSCs, Mini-Analysis software (Synaptosoft Inc, Decatur, GA). For quantification of synaptic inputs, the last 1-2 minutes or at least 80-100 events from control, drug and washout were used for statistical analysis. Changes in holding current were calculated as the difference between the current value immediately before drug perfusion and the maximum change in current induced by the drug. Majority of the neurons had an initial holding current close to 0pA (voltage hold

of -60mV), however due to the perfusion of some drugs which could induce changes in holding current for control period, only experiments in cells with a holding between 100 and -100pA were considered for analysis of changes in holding current. Outward currents that were defined as increases in positive holding current and inward currents that were considered as increases in negative holding current, were use throughout this thesis to report shifts in holding current. Current-voltage relationships were constructed for drug-induced currents by subtracting the voltage step values from control from those obtained after drug perfusion. Linear regression of these values was used to calculate the reversal potential of drug-induced currents (X-intercept when Y=0.0). Voltage-current relationships were used to calculate input resistance before and after drug perfusion which was considered as the slope of the linear regression trendline equation for these values. For maximum firing output, the current steps eliciting the highest firing frequency during control and drug conditions were compared. Single action potential mAHP amplitude was considered as the difference between the resting voltage before injecting the 10ms-long depolarizing current and the peak value of the mAHP (averaged across 15 traces). For the analysis of action potential half-width and rise-time, traces were not averaged to avoid errors due to action potentials being slightly out of phase when peak time was compared. Instead, 3 or more individual spikes were used for analysis. For each spike a differential function (dV/dt) was calculated and the point where dV/dt reached 10 mV/ms was used for the estimation of half-width and rise time. The obtained values from the 3 or more action potentials were then averaged and used for statistical comparisons. The half-width and rise time values from the different spikes within the same protocol file did not differ more than 1.5%. The voltage threshold for

action potentials was calculated from the first action potential elicited by current RAMPs using the differential method described above.

Drug washouts could not be performed in all the experiments in some sections and therefore were not used for statistical analysis. In single cell experiments from intact spinal cords this was due to the technical difficulty of recording from MNs during long periods of time. In recordings from spinal cord slices, washouts were not considered for statistical analysis of mAHP, firing output and action potential half-width due to the duration of some of the experiments (≥50 min) interfering with the quality of the data obtained for these parameters.

Data from ventral root bursting was analysed offline using DataView software (courtesy of Dr W. J. Heitler, University of St Andrews). Bursts were identified from the integrated/rectified trace from which frequency and duration were measured whereas amplitude was calculated from the respective segment of raw trace. Burst regularity was analysed as burst frequency variance. Data were averaged in 0.5-min time bins and normalized to a 10min pre-control period to construct time course plots. Statistical comparisons were made on raw data averaged over 5-min periods in each condition.

Data are presented as mean±standard error. In patch-clamp experiments each "n" corresponds to one cell whereas in ventral root recordings it corresponds to one whole spinal cord preparation. Kolmogorov-Smirnov Goodness of Fit Test was used to test for normality. Repeated measures ANOVA followed by Tukey's post-hoc tests were used to test for statistically significant differences ($p<0.05$) when comparing more than 2 conditions for normally distributed populations. Friedman test followed by Dunn's post-test was used when comparisons were made between non-normally distributed

populations to test for statistical significance ($p<0.05$). Paired *t*-tests (parametric) or Wilcoxon matched pairs tests (non-parametric) were used for comparing differences in means between 2 conditions; drug and control ($p<0.05$).

4. RESULTS

4.1. Muscarinic modulation of locomotor network output and MN function

One of the main focuses of this thesis was to understand the role of muscarinic receptor activation on locomotor output. Rhythmic behaviours, such as those involved in locomotion, are dependent upon CPG circuits that are comprised of many different IN subtypes. INs of the mammalian locomotor CPG are predominantly located in the ventral horn of the spinal cord, where they can generate patterns of activity required for adequate locomotor behaviours. The strength, or intensity, of the locomotor output is largely defined by MN firing, which in turn translates into the ventral root output signal that reaches the NMJs and leads to muscle contraction. Recordings of locomotor bursts from ventral nerve roots during fictive locomotion provide a valuable readout of the functional properties of CPG INs and MNs during rhythmic patterns of motor activity. In order to understand how M2 and M3 muscarinic receptors affect locomotor network output, muscarinic receptor antagonists were applied to whole spinal cord preparations while drug-induced locomotor activity was recorded from ventral roots and single-cell activity was recorded from MNs (sections 4.1.1-4.1.2).

After accessing the network effects of M2 and M3 receptor activation during fictive locomotion, the range of potential modulatory actions of muscarinic receptor activation was next studied at a single cell level. Given the lack of a clear marker for essential rhythmogenetic INs of the locomotor CPG and the impractical nature of systematic analysis of all genetically-defined ventral horn INs, the current study did not directly address the range of potential modulatory actions of muscarinic receptor activation on ventral horn INs. Instead, the cellular mechanisms of muscarinic receptor activation on MN function, and thus the intensity of locomotor output, were explored by

performing whole-cell patch-clamp recordings from MNs in isolated neonatal mouse spinal cord slices (sections 4.1.3-4.1.5).

The effects of muscarinic receptor activation were investigated by performing ventral root and single-cell recordings from MNs. Since ACh is released during locomotion (Dai *et al.*, 2009; Jordan *et al.*, 2014), muscarinic receptor antagonists were perfused during fictive locomotion to investigate their endogenous roles in the control of spinal motor networks. In spinal cord slices the general muscarinic receptor agonist, muscarine (10μM), was bath applied to activate muscarinic receptors to investigate their cellular mechanisms of action. Given that both the M2 and M3 receptors have been implicated in muscarinic-related actions on mammalian locomotor networks (Jordan *et al.*, 2014) the effects of muscarinic receptor activation on the properties of spinal neurons were then tested in the presence of either the M2 antagonist, methoctramine (10μM), or the M3 receptor blocker, 4-DAMP (2μM). In spinal cord slices, muscarine was also bath applied in the presece of both 4-DAMP and methoctramine to reveal the possibility of any non-M2/M3 receptor-mediated effects on MN function.

Single cell analysis of the effects of muscarinic receptor activation included investigation of subthreshold currents, neuronal output and synaptic transmission. Assessment of the effect of muscarinic receptor activation on the subthreshold properties of MNs was conducted by evaluating changes in holding current and by using current-voltage steps to infer any changes in input resistance. The effects of muscarinic receptor activation on neuronal output were studied by investigating repetitive firing parameters, particularly maximum firing elicited in control and in the presence of the muscarinic drugs. Changes in the mAHP amplitude were also analysed. For synaptic analysis, a mixture of PSCs was recorded. To understand if muscarine-mediated

changes in synaptic activity most likely involve changes in the excitability and firing activity of premotor networks or a direct modulation of last-order synaptic connections, muscarinic receptor drugs were perfused in the presence of TTX and mPSCs were recorded.

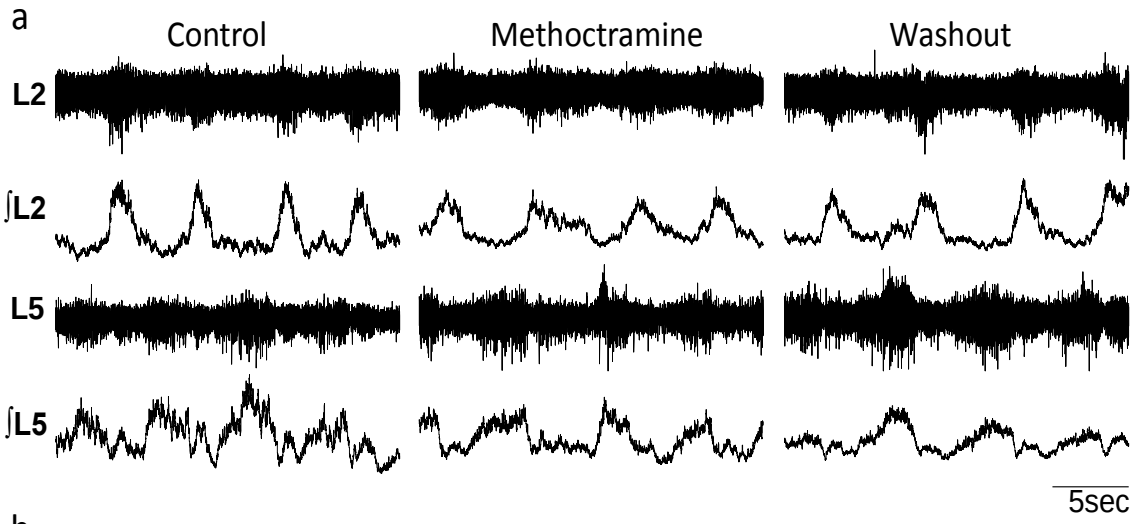
In summary, the work in this section of the thesis focused on determining the role of M2 and M3 muscarinic receptors in controlling the output of spinal motor circuitry during fictive locomotion and on the characterization of the cellular mechanisms underlying their modulation of MN function.

4.1.1. M2 muscarinic receptor blockade effects the regularity and reduces the amplitude of drug-induced locomotor output

Fictive locomotion was induced via the application of NMDA (5μM), 5-HT (10μM) and DA (50μM) and locomotor output was recorded through suction electrodes attached to the ventral roots. To address the role of M2 muscarinic receptors during locomotor-like bursting the selective antagonist methoctramine (10μM) was perfused. As illustrated in figure 4.1, blockade of M2 receptors with methoctramine significantly reduced burst frequency variance (control: $7.528\times10^{-3}\pm2.280\times10^{-3}Hz^2$; methoctramine: $2.224\times10^{-3}\pm4.831\times10^{-4}Hz^2$; washout: 9.381E-3$\pm2.228\times10^{-3}Hz^2$; n=24; $p<0.05$ Friedman's test with Dunn's post-hoc), reduced burst amplitude (control: 1.180±0.225; methoctramine:0.967±0.158; washout: 0.963±0.186; n=24; $p<0.05$ Friedman's test with Dunn's post-hoc), increased burst duration (control: 1454±137ms; methoctramine:1660±129ms; washout: 1259±134ms; n=24; $p<0.05$ Friedman's test with Dunn's post-hoc) and had no statistically significant effect on burst frequency

(control: 0.258±0.019Hz; methoctramine: 0.227±0.001Hz; washout: 0.283±0.023Hz; n=24).

The finding that blockade of M2 muscarinic receptors decreased burst frequency variance suggests that these receptors might have a role in destabilizing the regularity of the rhythmic bursts. Along with the reported increase in burst duration by methoctramine, which could involve modulation of CPG INs (Gosgnach *et al.*, 2006), these results highlight M2 receptor-mediated actions that are likely to occur at the level of INs that shape rhythmic output. The decrease in burst amplitude during fictive locomotion suggests that M2 receptors might directly modulate MN output. In summary, the data from this section shows that M2 muscarinic receptors modulate the rhythm generating component as well as the strength of motor output.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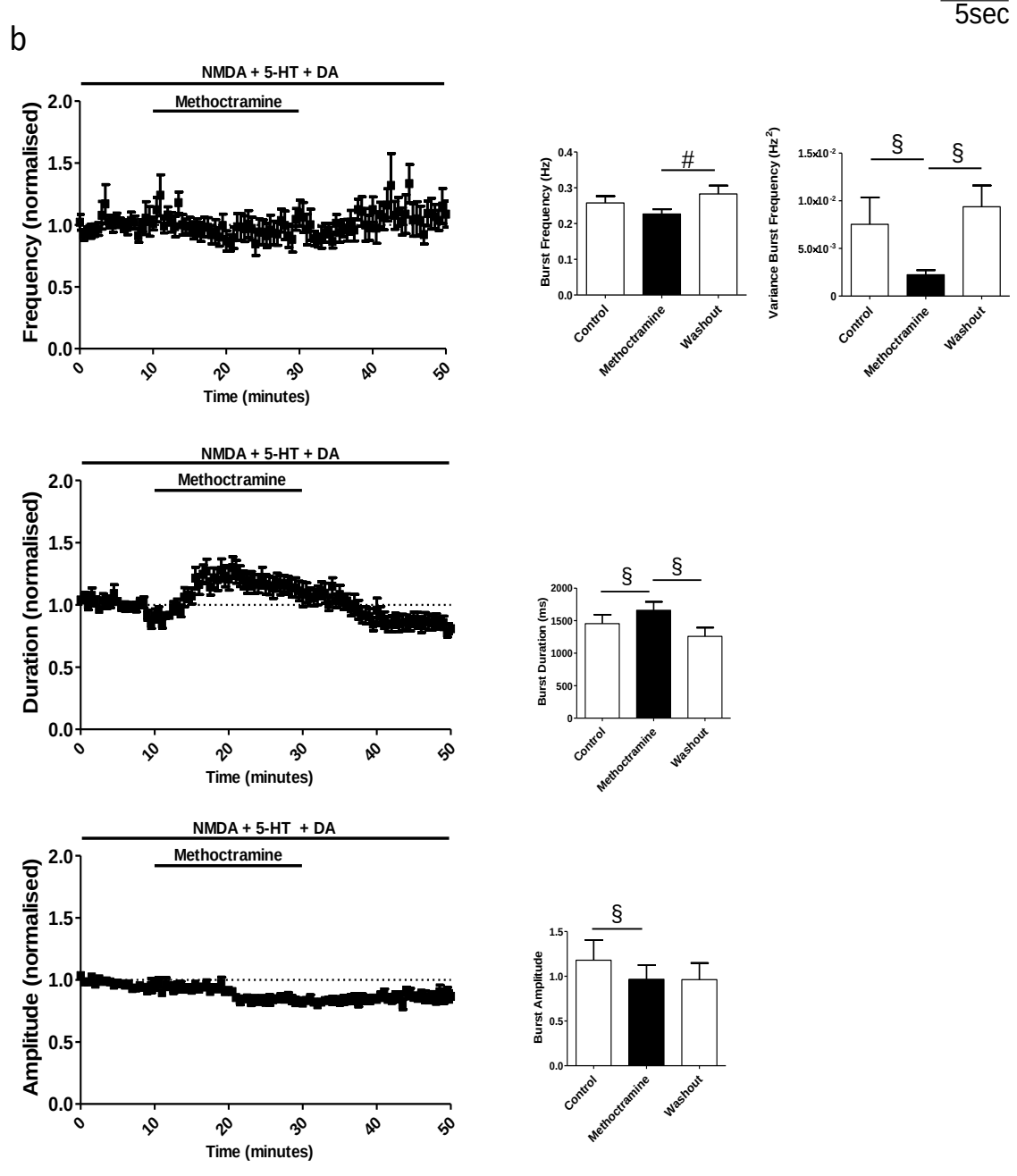
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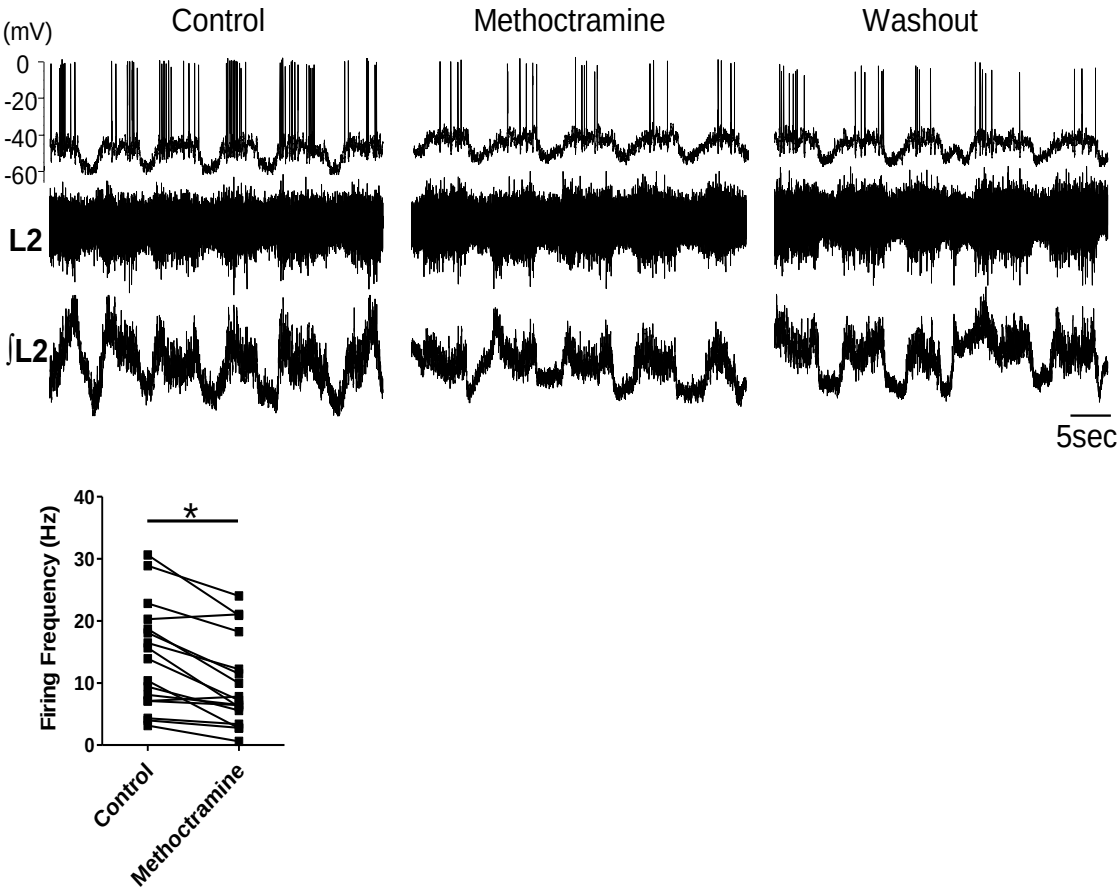
Figure 4.1 – M2 receptors modulate burst amplitude, duration and frequency variance during drug-induced locomotion. **(a)** raw (top) and integrated/rectified (bottom) traces with **(b)** averaged time course plots (left) and histograms of pooled data (right) illustrating the effects of methoctramine (10μM) on drug-induced locomotor output (n=24); #$p<0.05$ repeated measures ANOVA with Tukey's post-test; §$p<0.05$ Friedman's test with Dunn's post-hoc

To study if methoctramine-induced changes in rhythmic activity reflected a modulation of MN output, whole-cell patch clamp recordings were performed from lumbar MNs during locomotor output. The amplitude of locomotor-related bursts recorded from ventral roots reflects the overall output generated by MNs whose axons project through the roots. Therefore, the decrease in ventral root burst amplitude observed in the presence of methoctramine would be expected to reflect a reduction in MN firing frequency during bursting. As illustrated in figure 4.2, during recordings from bursting MNs in intact spinal cord preparations, blockade of M2 receptors significantly decreased MN firing frequency (control: 14.05±2.05Hz; methoctramine: 9.83±1.74Hz; n=17; $p<0.05$ paired *t*-test). These results confirm that M2 receptor blockade causes a reduction in the firing activity of rhythmically active MNs, which would account for the decrease in ventral root amplitude upon M2 muscarinic receptor blockade.



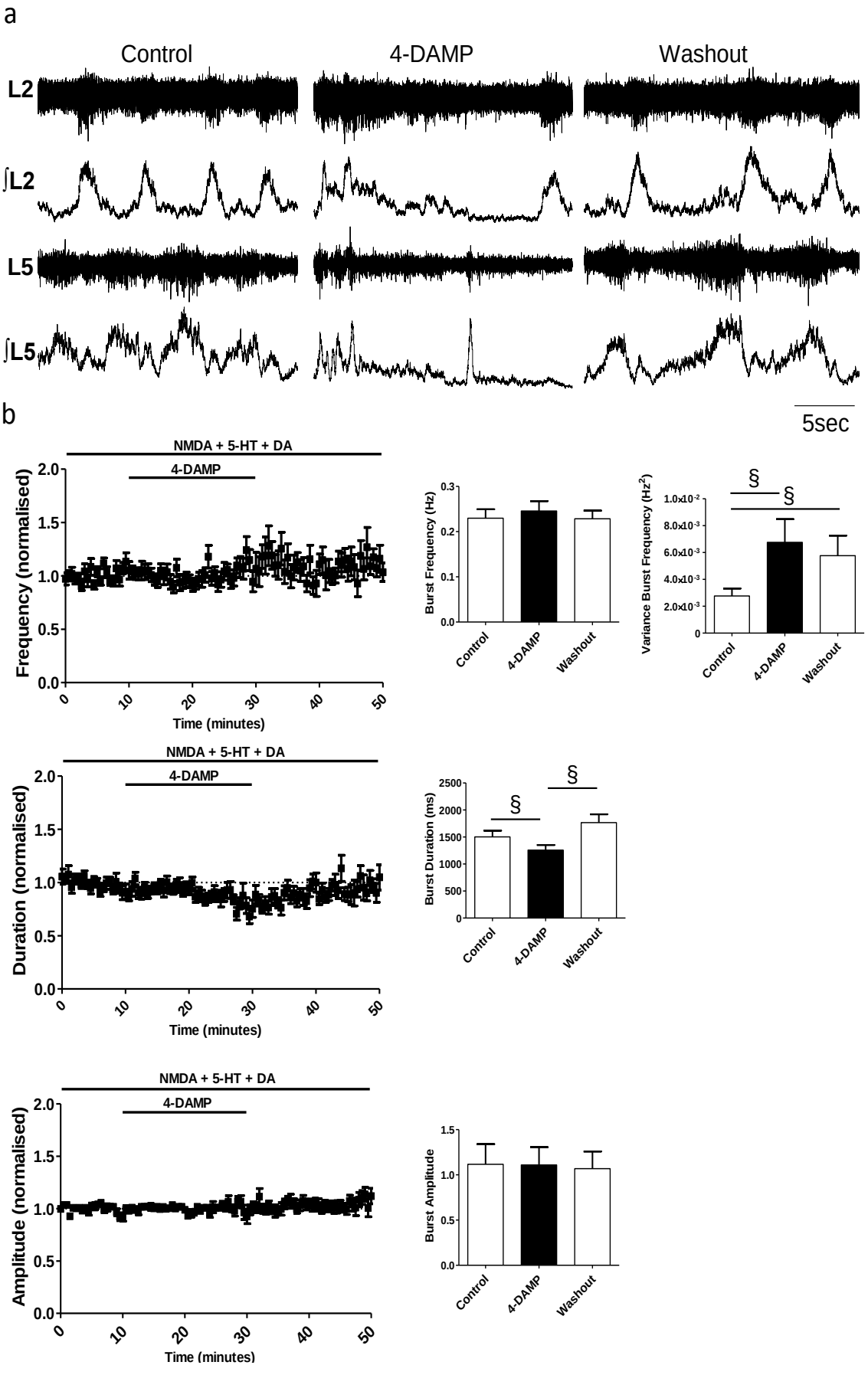
4.2 – M2 muscarinic receptor decreases burst amplitude by reducing MN firing during fictive locomotion. whole-cell patch-clamp recording from a bursting MN (top), raw (middle) and integrated/rectified (bottom) traces with firing frequencies plotted for all MNs tested showing the effects of methoctramine (10μM, n=17); *p<0.05 paired *t*-test

4.1.2. M3 muscarinic receptor blockade disrupts drug-induced locomotor output

To characterize the role of M3 receptors in the locomotor network, the M3-receptor antagonist 4-DAMP (2μM) was perfused while recording drug-induced bursts of rhythmic activity from lumbar ventral roots. Blockade of M3 receptors within spinal cord preparations (figure 4.3) had no significant effects on burst frequency (control: 0.230±0.020Hz; 4-DAMP: 0.246±0.022Hz; washout: 0.228±0.018Hz; n=22) or burst amplitude (control: 1.116±0.224; 4-DAMP: 1.110±0.197; washout: 1.068±0.190; n=22).

However, M3 receptor blockade increased burst frequency variance (control: $2.765\times10^{-3}\pm5.541\times10^{-4}Hz^2$; 4-DAMP: $6.756\times10^{-3}\pm1.727\times10^{-3}Hz^2$; washout: $5.760\times10^{-3}\pm1.493\times10^{-3}Hz^2$; n=22; $p<0.05$ Friedman's test with Dunn's post-hoc) and decreased burst duration (control: 1502±115ms; 4-DAMP: 1257±93ms; washout: 1765±153; n=22; $p<0.05$ Friedman's test with Dunn's post-hoc).

The finding that blockade of M3 receptors during fictive locomotion destabilized the locomotor rhythm by increasing the burst frequency variance and decreasing burst duration, indicate that M3 muscarinic receptors exclusively modulate rhythm generating neurons since there was no significant change on burst amplitude. These findings with 4-DAMP contrast the results obtained in the presence of methoctramine, highlighting that these receptors seem to have opposite actions on the modulation of spinal CPG circuits during locomotion.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4-DAMP
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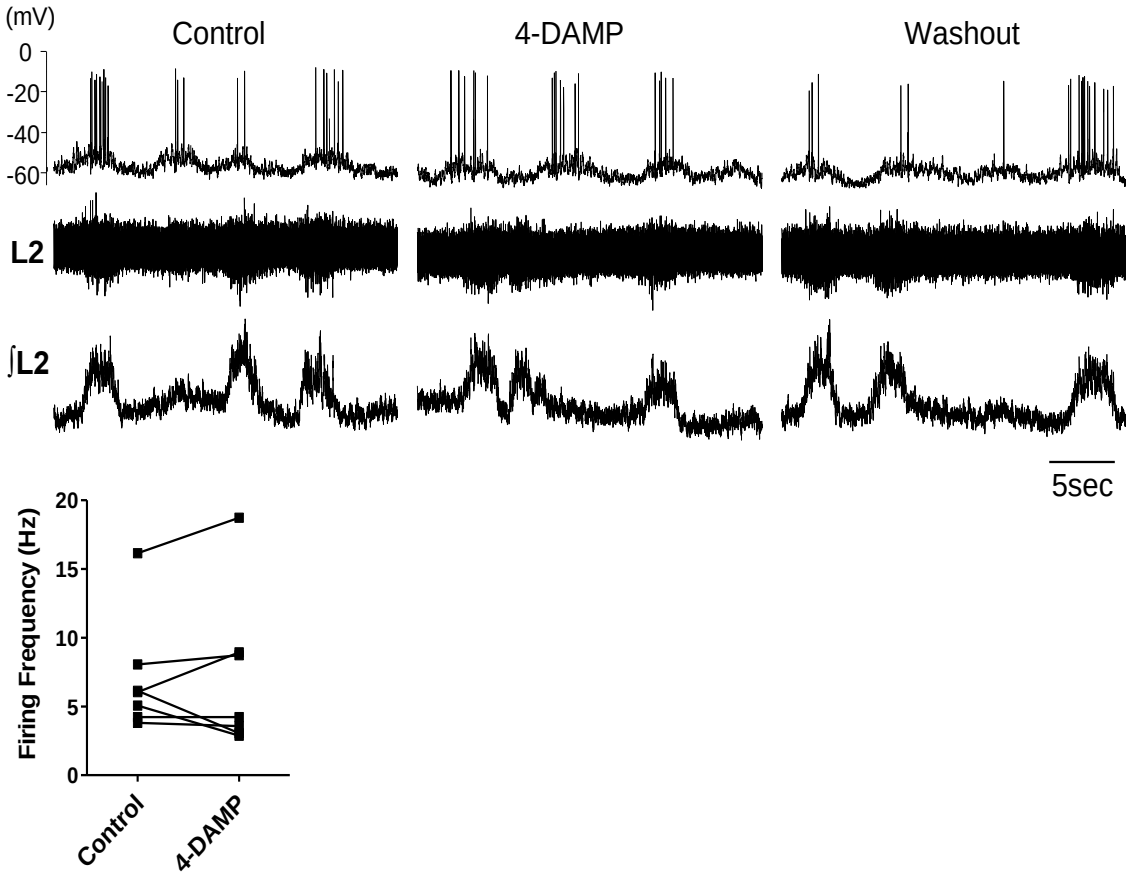
4-DAMP
Washout

Figure 4.3 – M3 receptors modulate locomotor rhythm stability. **(a)** raw (top) and integrated/rectified (bottom) traces with **(b)** averaged time course plots (left) and histograms of pooled data (right) showing the effects of 4-DAMP (2μM) on drug-induced locomotor output (n=22); #$p<0.05$ repeated measures ANOVA with Tukey's post-test; §$p<0.05$ Friedman's test with Dunn's post-hoc

To explore if blockade of M3 muscarinic receptors would impact on the excitability of MNs during episodes of drug-induced locomotion, single cell activity was recorded during bursts of activity. As seen in figure 4.4, 4-DAMP had no significant effect on the firing frequency of MNs within intact spinal cord preparations (control: 7.07±1.61Hz; 4-DAMP: 7.16±2.16Hz; n=7). The apparent mismatch between MN activity and ventral root bursting in figure 4.4 in the presence of 4-DAMP is a clear example of the disruptive nature of M3 receptor blockade during fictive locomotion.

This demonstrates that M3 muscarinic receptor-mediated modulation during fictive locomotion is predominantly focused on the regulation of INs that comprise the CPG network. Activation of these receptors in CPG neurons might be important to stabilize the rhythm by conferring appropriate regularity to set adequate patterns of activity.



4.4 – Blockade of M3 muscarinic receptors during drug-induced locomotion does not affect MN firing. whole-cell patch-clamp recording from a bursting MN (top), raw (middle) and integrated/rectified (bottom) traces with firing frequencies plotted for all MNs tested illustrating the effects of M3 receptors blockade with 4-DAMP (2µM, n=7); $p>0.05$ Wilcoxon matched pairs test

4.1.3. Muscarine reveals both M2 receptor-mediated outward currents and M3 receptor-dependent inwards currents that vary dependent on MN size

To explore changes in neuronal excitability mediated by muscarinic receptors, subthreshold properties of MNs were first studied by evaluating changes in holding

current and input resistance upon muscarine application during voltage-clamp recordings.

Two different responses to muscarine were observed (figure 4.5a). In one subset of MNs, muscarine induced an inward current (-50.96±8.13pA n=20), which was associated with an increase in input resistance (control: 92.83±24.75MΩ, muscarine: 118.54±29.29MΩ, reversal -78.43mV, n=9). While in other MNs, muscarine elicited an outward current (43.32±15.57pA; n=10), which was associated with a decrease in input resistance (control: 134.38±20.14MΩ, muscarine: 105.96±19.71 MΩ, reversal -92.61mV, n=6). Interestingly, MNs in which an inward current was observed were larger, as indicated by whole-cell capacitance values (C_m=146±6pF, n=20), than those in which an outward current was induced (C_m=97±7pF, n=10).

To reveal if these muscarine-induced currents were due to direct activation of postsynaptic muscarinic receptors on MNs, muscarine was next perfused in the presence of TTX (figure 4.5b). Under these conditions, muscarine again induced an outward current in small MNs (35.80±7.67pA; C_m=91±6pF; n=11), which was associated with a decrease in input resistance (TTX: 51.70±10.31MΩ, TTX and muscarine: 57.68±10.98MΩ, reversal -90.18mV, n=6), while in larger MNs it caused an inward current (-42.86±13.57pA; C_m= 146±12pF; n=8) that was accompanied by an increase in input resistance (TTX: 64.14±8.87MΩ, TTX and muscarine: 53.17± 6.10 MΩ, reversal -76.08mV, n=6).

Changes in current in the presence of muscarine indicate that activation of muscarinic receptors preferentially depolarizes large and hyperpolarizes small MNs. These differences seem to involve modulation of muscarinic receptors that are expressed on MNs.

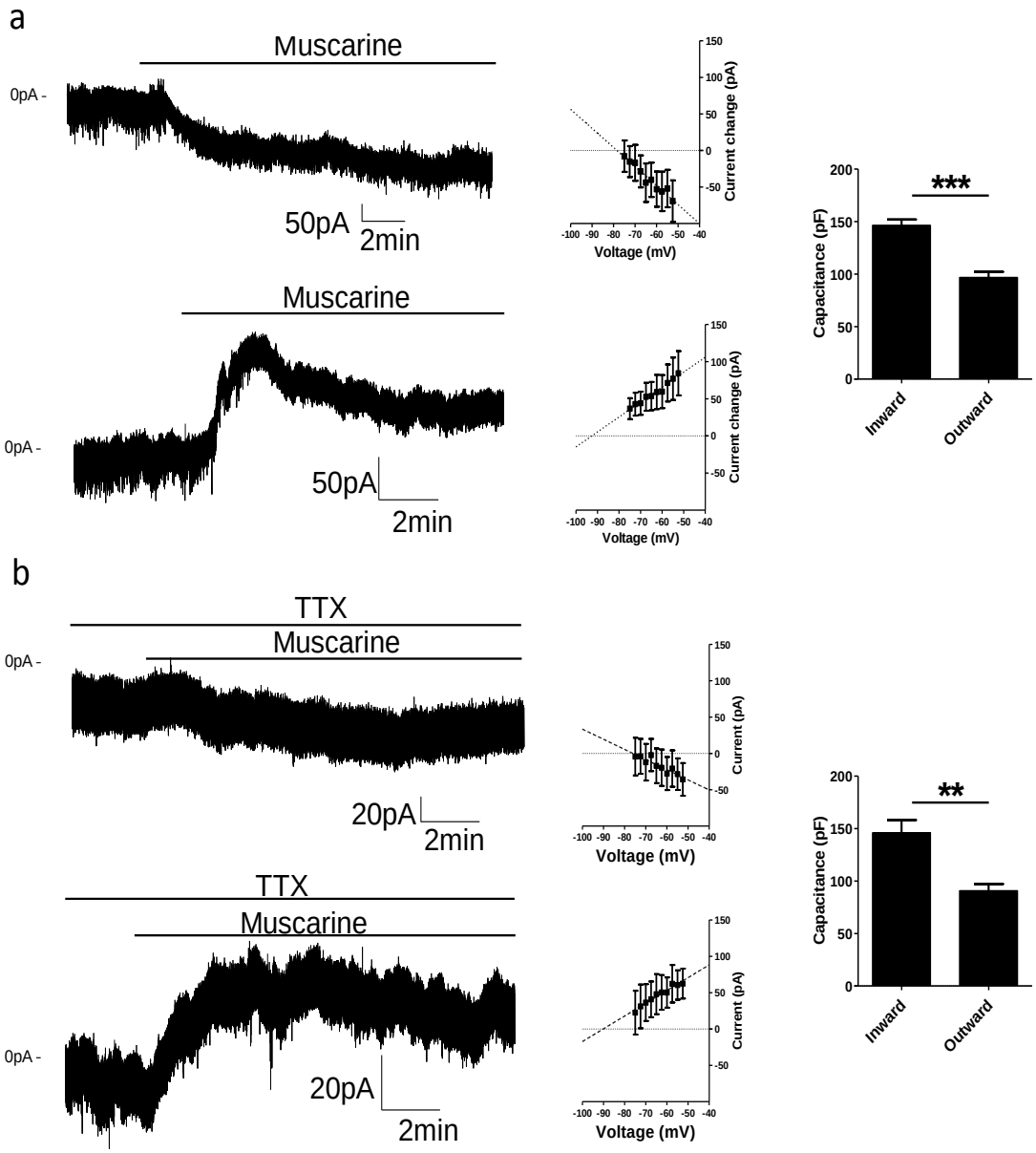


Figure 4.5 – Muscarine induces an inward current in large MNs and outward current in small MNs thought a mechanism involving last-order synapse muscarinic receptor modulation. **(a)** voltage clamp transient (left) with respective I-V plots (middle) and MN capacitance (right), illustrating inward (top, n=20) and outward (bottom, n=10) holding currents elicited by muscarine (10µM) that were accompanied by an increase (n=9) and decreased (n=6) in input resistance, respectively; **(b)** changes in holding current in the presence of TTX (0.5µM, left) with input resistance (middle) and MN size statistical comparison (right) illustrating the inward current (n=8) and increase in input resistance (n=6) in large MNs and a outward current (n=11) in smaller MNs that decreased cell resistance (n=6). *p<0.05**p<0.01***p<0.001 unpaired *t*-test

To understand which receptor subtypes are responsible for the muscarine-induced changes in holding current, the non-selective agonist muscarine was next perfused in the presence of the M2 receptor antagonist methoctramine or the M3 receptor antagonist 4-DAMP.

In the presence of methoctramine, muscarine elicited an inward current (-53.52±6.64pA, n=17) and increased input resistance (methoctramine: 90.90±3.78MΩ, methoctramine and muscarine: 104.68±3.52MΩ, reversal -116.10mV, n=8) (figure 4.6a). To check if these currents involved activation of postsynaptic muscarine receptors on MNs, experiments were performed when evoked transmission was blocked by TTX. As seen in figure 4.6b, activation of muscarinic receptors with methoctramine and TTX present still induced an inward current (-35.24±7.62pA, n=17) and also increased input resistance (TTX and methoctramine: 54.08±5.65MΩ, TTX, methoctramine and muscarine: 64.69±6.61MΩ, reversal -76.40mV, n=8).

Activation of muscarinic receptors with muscarine in the presence of methoctramine, revealed an inward current in all MNs tested. Since there was no evidence of an outward current when M2 receptors were blocked, these results indicate that M2 receptors are responsible for the hyperpolarizing currents previously demonstrated in MNs in response to muscarine application.

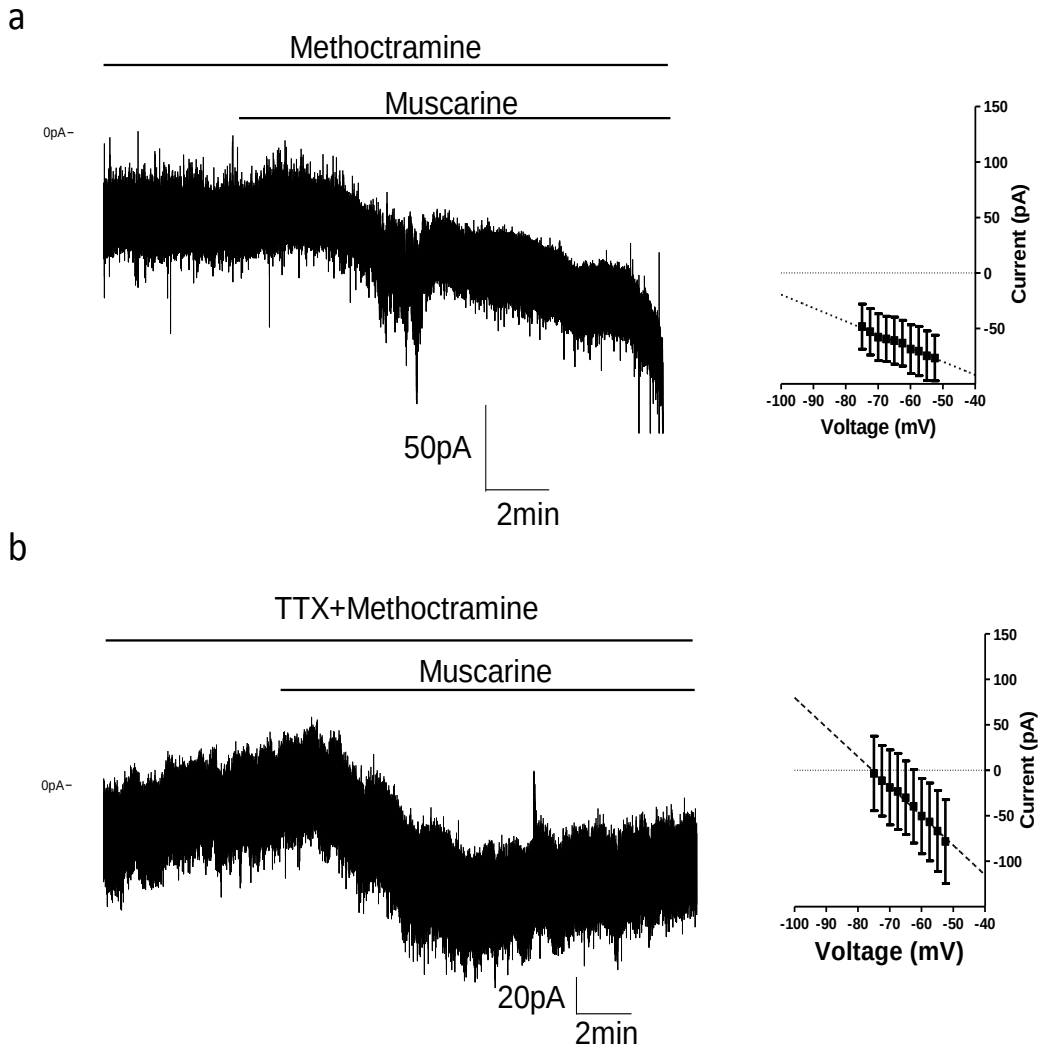


Figure 4.6 – Muscarine in the presence of M2 receptor antagonism induces an inward current on MNs. Voltage clamp representative traces (left) and I-V plots (right) illustrating that **(a)** muscarine (10µM) co-applied with the M2 selective antagonist (10µM) induced an inward current (n=17) leading to an increase in membrane resistance (n=8) even in **(b)** the presence of TTX (0.5µM, n=17 for left figure and n=8 for right figure).

To investigate whether M3 receptors might be responsible for the inward current induced by muscarine when M2 receptors were blocked, muscarine was next perfused with the M3 blocker 4-DAMP. Co-application of 4-DAMP and muscarine elicited an outward current (88.22±13.69pA, n=14). In a group of MNs this was associated with a decrease in input resistance (4-DAMP: 117.15±22.94MΩ, 4-DAMP and muscarine:

85.70±19.85MΩ, reversal -106.70mV, n=6) while in another subset of MNs input resistance was increased (4-DAMP: 66.68±5.18MΩ, 4-DAMP and muscarine: 81.21±6.9MΩ, reversal -71.86mV, n=9) (figure 4.1.7a). There was no significant difference in capacitance between both groups of MNs (MNs with increased input resistance: 141±10pF, n=9; MNs with decreased input resistance: 132.12±16pF, n=6). When TTX was bath applied, muscarine in the presence of 4-DAMP still induced an outward current (47.62±16.94pA, n=15) and increased input resistance in a subset of MNs (TTX and 4-DAMP: 82.40±11.13MΩ, TTX, 4-DAMP and muscarine: 72.37±10.53MΩ, reversal -79.85mV, n=6) while in another subgroup of MNs it decreased input resistance (TTX and 4-DAMP: 69.94±9.54MΩ, TTX, 4-DAMP and muscarine: 75.87±10.77MΩ, reversal -94.56mV, n=6) (4.1.7b). There was again no significant difference in capacitance between the MNs with increased (133.63±6.86pF, n=6) and decreased input resistance (107.99±12.23pF, n=6).

Since no inward currents were induced by muscarine in the presence of 4-DAMP, these results suggest that M3 receptors are responsible for the muscarine-induced inward currents revealed earlier in MNs. The increase and decrease observed in input resistance when muscarine was co-perfused with 4-DAMP suggests that activation of M2 receptors results in different changes in input resistance. These data indicate that M2 receptor activation might have variable effects on ion channels in different MNs.

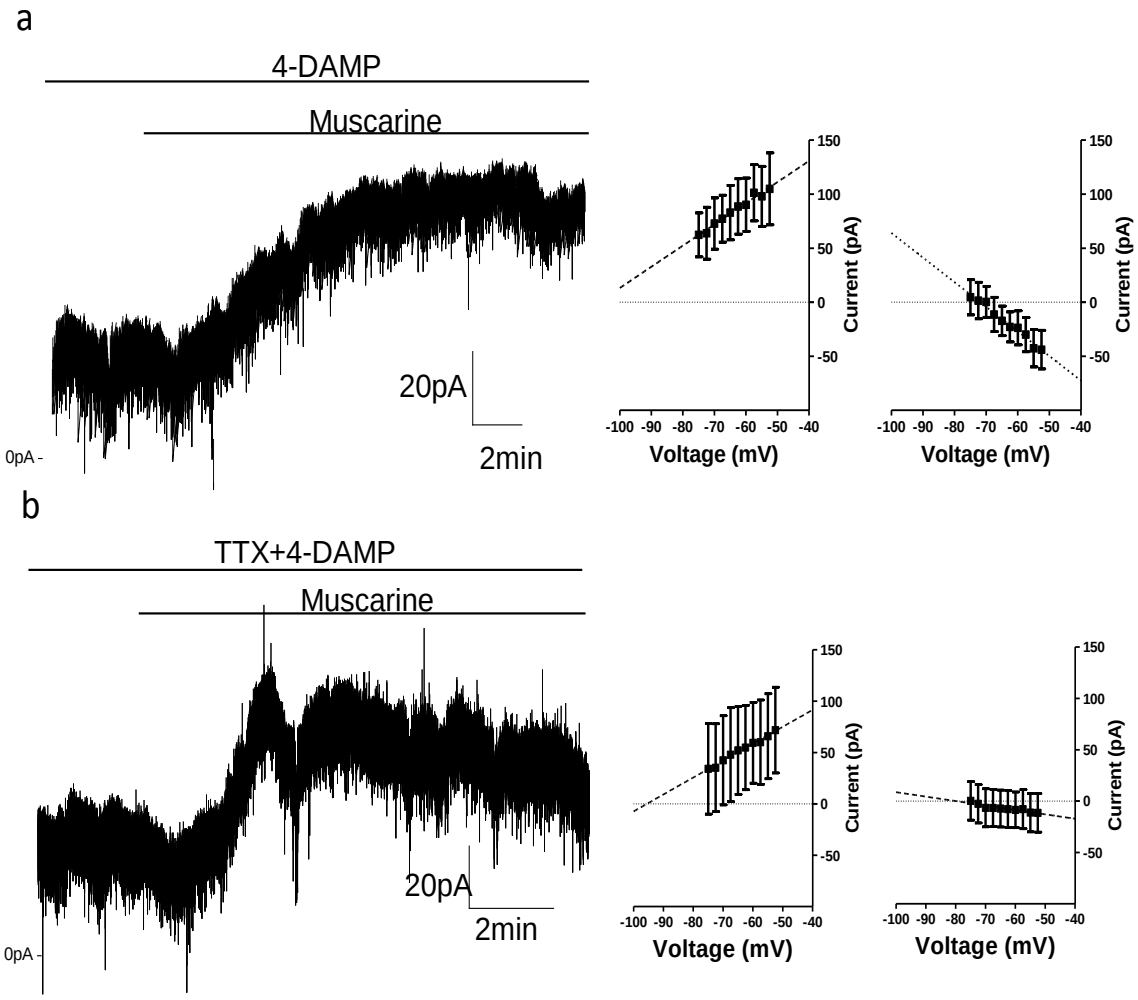


Figure 4.7 – Muscarine in the presence of 4-DAMP induces a outward current on MNs. Voltage clamp representative traces (left) and I-V plots (right) illustrating that (**a)** muscarine (10µM) in the presence of 4-DAMP (2µM) elicited an outward current (n=14) and in a group of MNs decreased (n=6) while in other MNs it increased input resistance (n=9) and **(b)** when TTX (0.5µM) was applied, muscarine with 4-DAMP also elicited an outward current on MNs (n=15) and decreased (n=6) or increased (n=6) input resistance in different MNs.

Next, to address the potential role of endogenous ACh in the induction of currents in MNs, the effects of each of the muscarinic receptor subtype antagonists on holding current were explored. Application of methoctramine induced an inward current in MNs (-38.57±6.21pA, n=18) and increased input resistance (control: 70.67±7.92MΩ, methoctramine: 82.50±7.07MΩ, reversal -79.54mV, n=8) (figure 4.8a). 4-DAMP induced an outward current (51.95±15.00pA, n=17) and a decrease in input resistance in

MNs (control: 89.50±7.60MΩ, 4-DAMP: 72.75±7.22MΩ, reversal -82.68mV, n=7) (figures 4.8b).

These data show that blockade of M2 receptors removes a tonic outward current while antagonism of M3 receptors removes a tonic inward current. The results further indicate that M2 receptors might modulate a change in current, which would result in MN hyperpolarization, whereas M3 receptors modulate an inward current that would depolarize MNs. M2 and M3 muscarinic receptors have opposite actions on the modulation of subthreshold properties pointing out the possibility of a reciprocal interaction on the regulation of MN function.

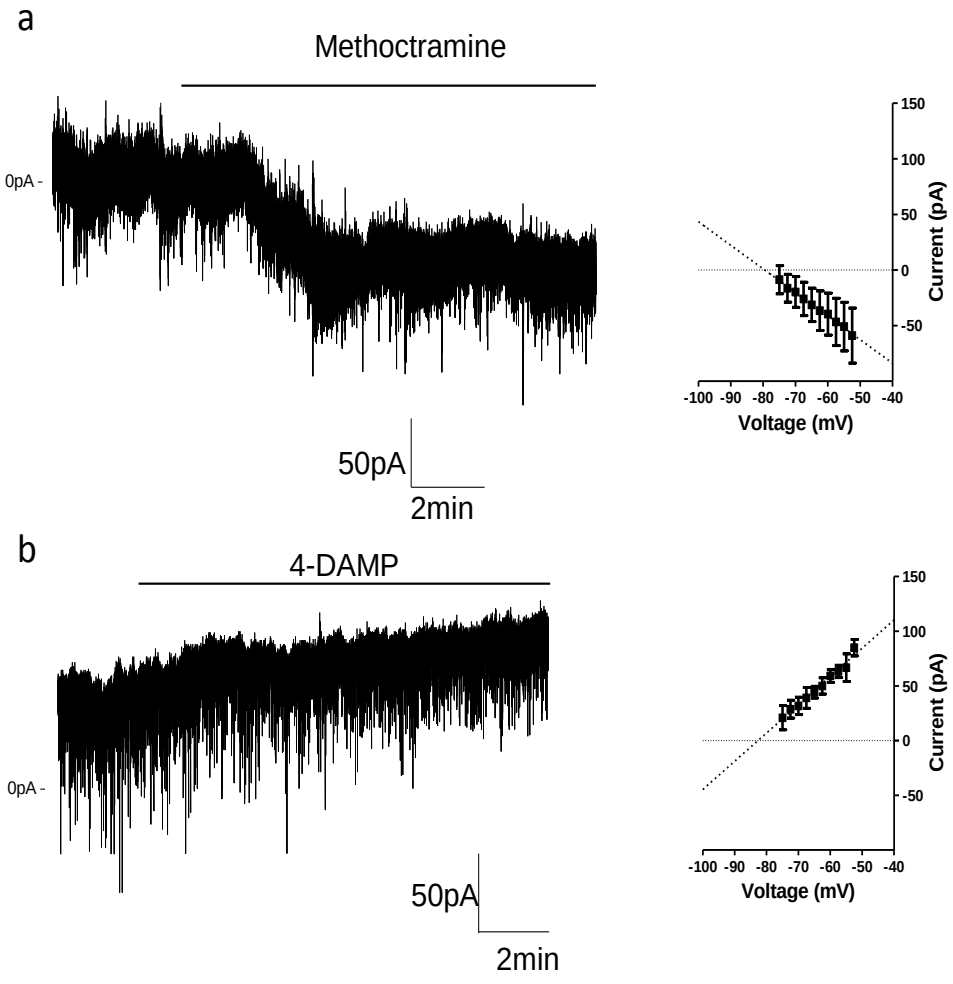


Figure 4.8 – Methoctramine induces an inward and 4-DAMP elicits an outward current on MNs. Voltage clamp representative traces (left) and I-V plots (right) illustrating that **(a)** 4-DAMP (2μM) induced an outward current (n=18) and increased input resistance

(n=7) and **(b)** methoctramine (10µM) caused an inward current on MNs (n=17) and increase input resistance (n=8).

To determine whether if any non-M2/M3 receptors were involved in the previously described muscarinic receptor actions, the non-selective agonist muscarine was perfused in the presence of both methoctramine and 4-DAMP while recording from MNs. As seen in figure 4.9, in the presence of methoctramine and 4-DAMP, muscarine had no effects on holding current (-1.0±8±6.17pA, n=10) or input resistance (4-DAMP and methoctramine: 97.13±14.21MΩ, 4-DAMP, methoctramine and muscarine: 95.31±17.81MΩ, n=7).

Since muscarine did not have any significant effects on MNs in the presence of methoctramine and 4-DAMP, these results suggest that M2 and M3 receptors in spinal cord slices are solely responsible for the muscarinic modulation of spinal motor function which is in agreement with previous evidence from the rat spinal cord (Jordan *et al.*, 2014).

a

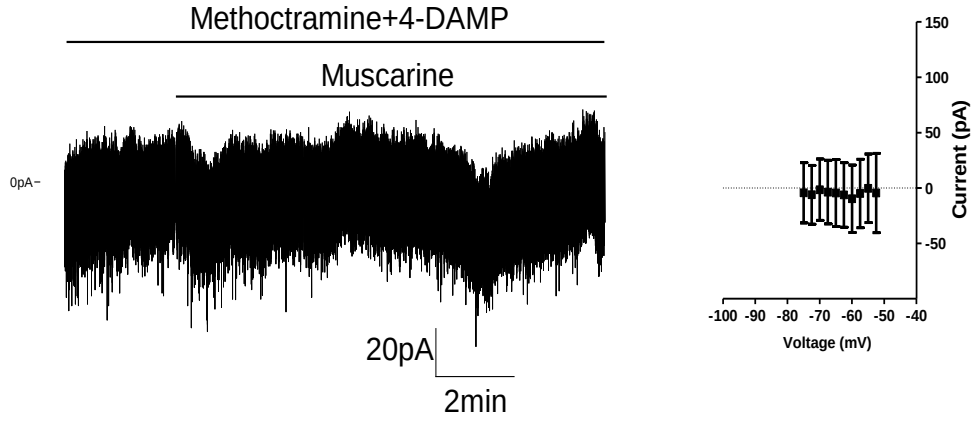


Figure 4.9 – Muscarine affects MN excitability exclusively through M2 and M3 receptors activation. Voltage-clamp transient showing no current change by muscarine in the presence of 4-DAMP (2µM) and methoctramine (10µM, left, n=10) and respective input resistance relationship (right, n=7).

4.1.4. Muscarine increases MN maximum firing output via activation of both M2 and M3 muscarinic receptors

To investigate the modulatory effects of muscarinic receptor activation on MN firing output, muscarine was perfused while action potentials (repetitive or single spikes) were elicited in MNs via current injection in current-clamp mode.

First, the effects of muscarinic receptor activation on repetitive MN firing were assessed. Repetitive firing was induced using a series of current steps of increasing magnitude. Comparisons of repetitive firing elicited in control conditions and in the presence of muscarine revealed that muscarine receptor activation increased MN maximum firing (control: 32.24±2.69Hz; muscarine: 35.01±2.80Hz; n=14; $p<0.01$ paired *t*-test; figure 4.10a).

Given previous reports that muscarine receptor activation affects MN firing by modulating mAHPs (Miles *et al.*, 2007), muscarine was next applied during short current steps which elicited only single action potentials followed by a clear mAHP. These single action potential protocols showed that muscarine significantly decreased mAHP amplitude (control: -4.93±1.08mV; muscarine: -2.96±0.82mV; $p<0.05$ Wilcoxon matched pairs test) (figure 4.10b).

These data confirm that muscarinic receptor activation increases MN output and decreases mAHP amplitude, as previously described (Miles *et al.*, 2007). The activation of muscarinic receptors resulted in an overall increase in MN spiking output. The reduction in mAHP amplitude could contribute to augmented firing which was a feature associated with M2 receptor activation (Miles *et al.*, 2007). Experiments utilising muscarinic receptor antagonists were next performed to investigate the contribution of M2 and M3 muscarinic receptors to the muscarinic modulation of MN firing.

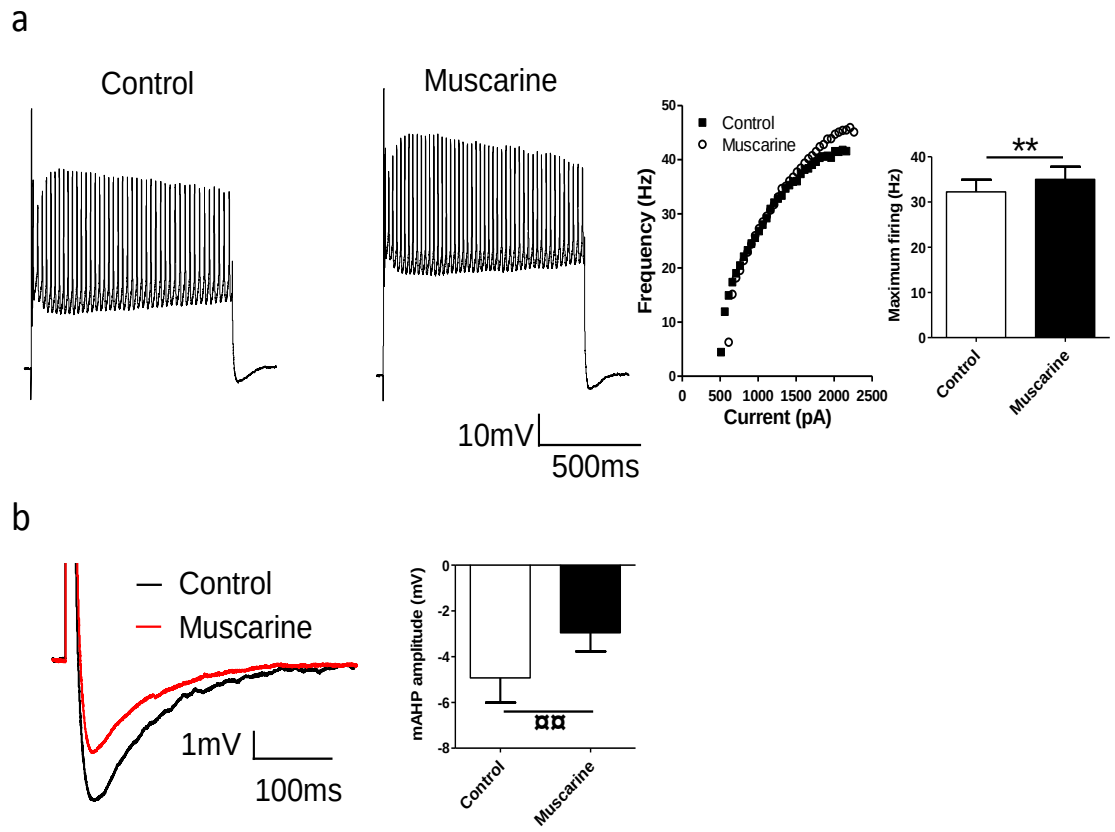


Figure 4.10 – Muscarine increases MN maximum firing output and decreases mAHP amplitude; **(a)** representation of increased MN maximum firing in response to increments of current injection in the presence of muscarine (10µM, left) with respective f-I plot (middle) and averaged maximum firing (right, n=14); **(b)** truncated single actions potentials showing the effects of muscarine on mAHP amplitude (n=17). **p<0.01 paired *t*-test. ¤ ¤p<0.01 Wilcoxon matched pairs test

To address the role of M2 receptors in modulating MN firing properties, MN output was studied during perfusion of muscarine in the presence of the M2 receptor antagonist methoctramine. As illustrated in figure 4.11a, muscarine did not change MN maximum firing in the presence of methoctramine (methoctramine: 28.76±1.58Hz; methoctramine and muscarine: 29.69±2.00Hz; n=11). The M2 antagonist also effectively blocked the muscarine-dependent reduction in mAHP amplitude

(methoctramine: -4.65±1.16mV; methoctramine and muscarine: -3.58±0.77mV; n=11) (figure 4.11b).

The absence of muscarine-induced changes in maximum firing and mAHP amplitude with methoctramine indicate that M2 muscarinic receptors are important mediators of cholinergic modulation of MN output, as previously suggested (Miles *et al.*, 2007). In addition, these results indicate that the activation of M3 receptors, in the presence of M2 receptor blockade, is insufficient to increase MN output.

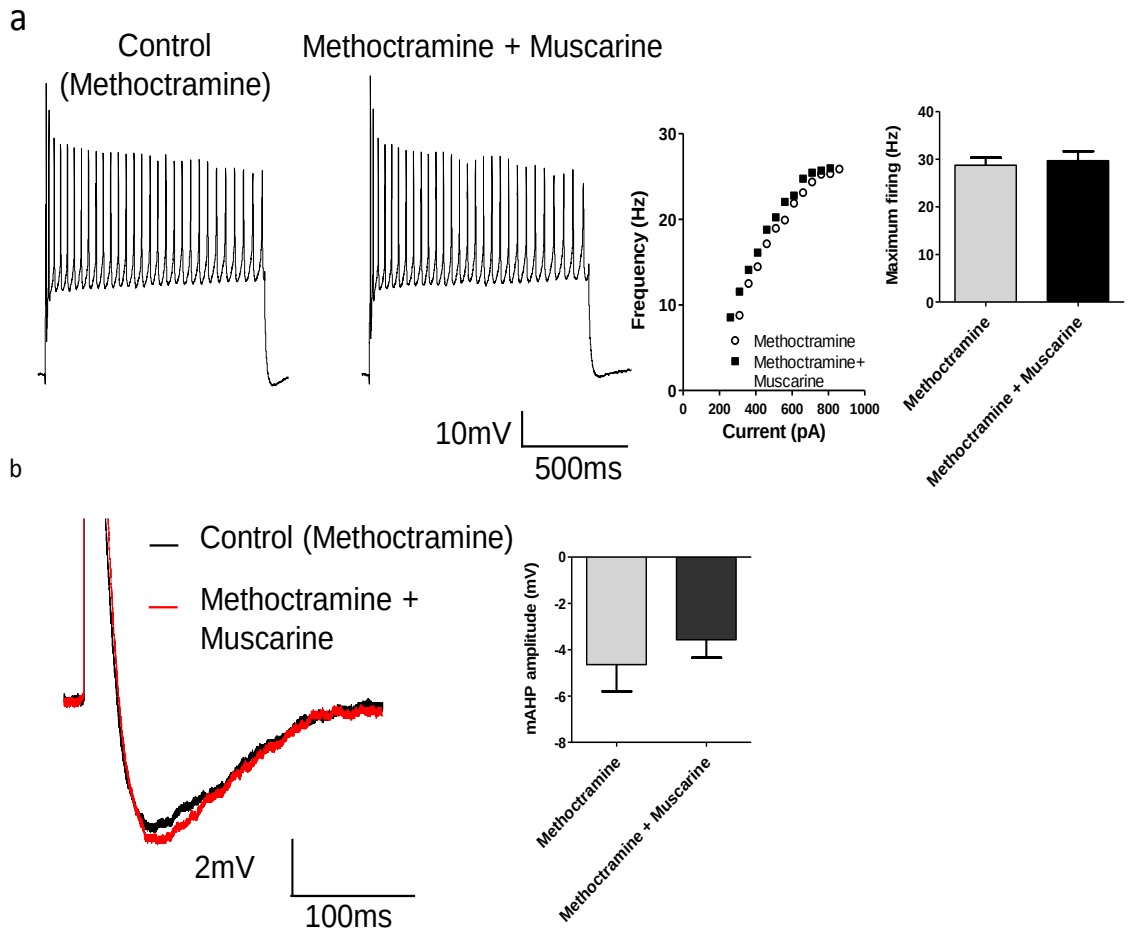


Figure 4.11 - M2 receptor blockade removes the effect of muscarine on MN maximum firing. **(a,c)** step of current (left) with respective f-I plot (middle) and averaged maximum firing (right) and **(b,d)** truncated single actions potentials (left) with mean mAHP amplitude plots (right) illustrating the recorded effects of the presence of muscarine (10µM) co-perfused with methoctramine (10µM) on firing output (n=11) and mAHP (n=11). p>0.05 paired t-test and p>0.05 Wilcoxon matched pairs test.

To reveal the potential impact of M3 receptors on MN firing, muscarine was next perfused with 4-DAMP and changes in maximum firing rates and the mAHP were studied. Blockade of M3 receptors prevented the muscarine-induced increase in maximum firing (4-DAMP: 29.32±1.40Hz; 4-DAMP and muscarine: 27.92±1.73Hz; n=15) but not the muscarine-induced decrease in mAHP amplitude (4-DAMP: -5.54±0.55mV; 4-DAMP and muscarine: -3.17±0.67mV; n=10; $p<0.05$ paired *t*-test) (figure 4.12).

Blocking M3 receptors removed the increase in maximum firing but did not affect the decrease in mAHP amplitude induced by muscarine, suggesting that these receptors may play a role in modulating MN excitability via a mechanism that does not target the mAHP. Surprisingly, both M2 and M3 receptor antagonists effectively prevented the muscarine-mediated increase in maximum firing. This perhaps indicates that a balance between M2 and M3 receptor activation is required for the reported cholinergic increase in output. These data show that both M2 and M3 muscarinic receptors are important in cholinergic modulation of MN firing.

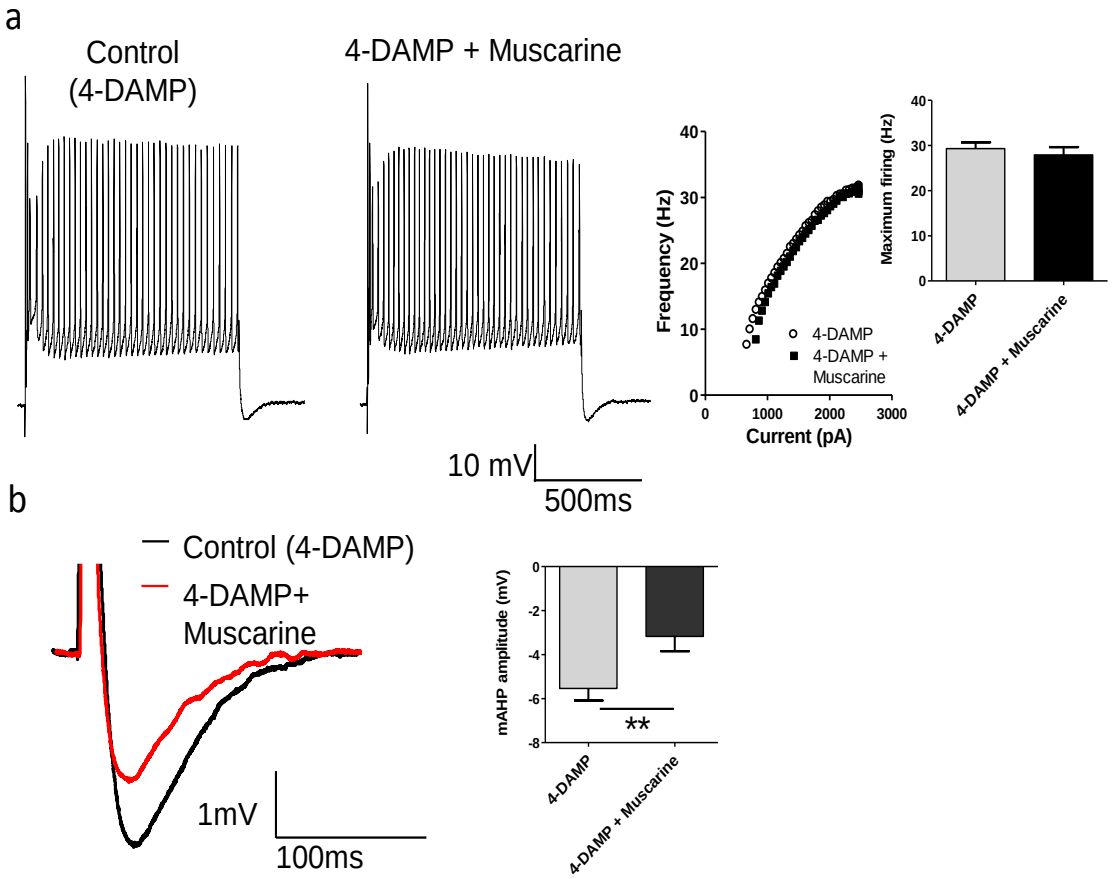


Figure 4.12 - M3 muscarinic receptors are involved in muscarine-induced increase in MN output. **(a,c)** injected step of current (left) with respective current-frequency plot (middle) and averaged maximum firing (right) and **(b,d)** truncated single actions potentials with mean mAHP amplitude plots (left) exemplifying the recorded effects in the presence of 4-DAMP (2µM) co-perfused with muscarine (10µM) on firing output (n=15) and mAHP (n=10); **p<0.01 paired *t*-test.

To reveal the presence of any endogenous activation of M2 and M3 muscarinic receptors that could underlie tonic cholinergic modulation of MN firing properties, selective antagonists were perfused and MN output was studied. Methoctramine application did not affect MN maximum firing (control: 29.80±1.80Hz; methoctramine: 28.59±1.70Hz; n=14) nor mAHP amplitude (control: -4.74±0.47mV; methoctramine: -4.63±0.57mV; n=17) (figures 4.13a-b). Similarly, 4-DAMP had no significant effects on MN maximum firing (control: 30.04±2.11Hz; 4-DAMP: 27.89±2.43Hz; n=11) nor mAHP amplitude (control: -5.92±0.66mV; 4-DAMP: -5.81±065mV; n=12) (figures

4.13c-d). There could be changes in overall cell resistance noticeable during the current step (see figures 4.13a and 4.13c) which could affect neuronal excitability, however no significant change in averaged maximum firing output were observed.

These results indicate that blockade of M2 or M3 muscarinic receptors does not affect MN output indicating that activation at rest of these receptors does not significantly modulate MN firing in spinal cord slice preparations.

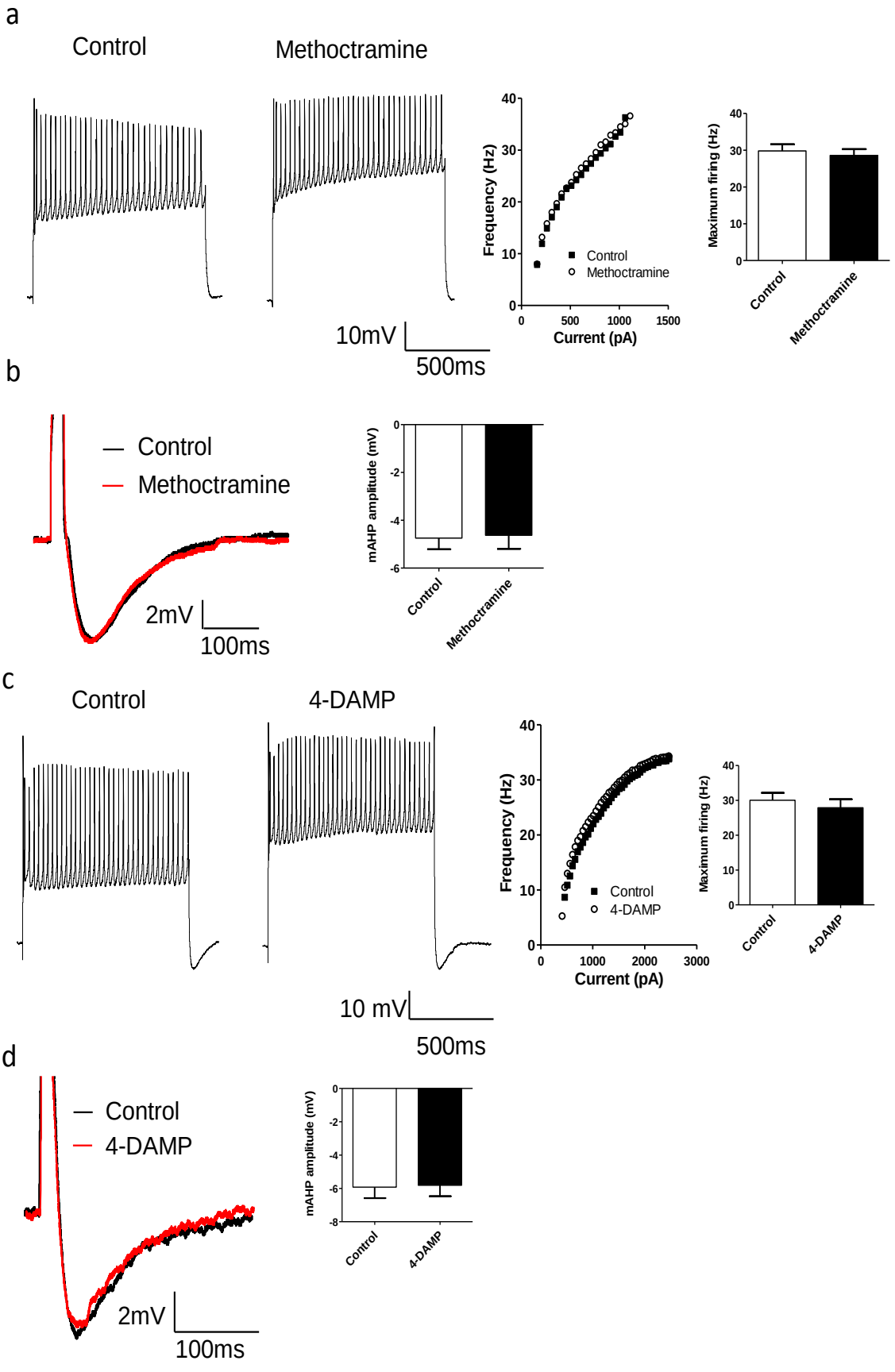

a
Control
Methoctramine
10mV
500ms
Frequency (Hz)
Current (pA)
Control
Methoctramine
Maximum firing (Hz)
b
Control
Methoctramine
2mV
100ms
mAHP amplitude (mV)
c
Control
4-DAMP
10 mV
500ms
d
Control
4-DAMP
2mV
100ms

Figure 4.13 - M2 and M3 muscarinic receptor blockade does not affect MN output **(a,c)** step of current (left) with respective f-I plot (middle) and averaged maximum firing (right) and **(b,d)** truncated single actions potentials (left) with mean mAHP amplitude plots (right) illustrating the recorded effects of the presence of methoctramine (10μM) on firing (n=14) and mAHP (17) and exemplifying the recorded effects in the presence of 4-DAMP (2μM) on MN maximum output (n=11) and mAHP (12).

4.1.5. M2 and M3 muscarinic receptors modulate synaptic inputs to MNs

The effects of M2 and M3 muscarinic receptor activation could also affect MN function by modulating synaptic inputs from spinal INs. To address this, mixed PSCs were recorded from MNs while muscarinic receptors were either activated or inhibited.

Muscarine was first bath applied to spinal cord slices while whole-cell patch-clamp recordings were performed from MNs to explore whether muscarinic receptor activation effected synaptic drive to MNs. As seen in figure 4.14a, muscarine had an initial excitatory (less than 10 minutes of perfusion) followed by a delayed inhibitory effect on the frequency of synaptic events. This was revealed by an initial decrease, followed by a later increase in inter-event interval (control: 559.08±84.54ms; muscarine before 10min: 290.68±63.53ms; muscarine 15-30min: 896.36±156.44ms; washout: 629.357±107.29ms; n=12; $p<0.05$ repeated measures ANOVA with Tukey's post-test). Muscarine also caused a transient increase in the amplitude of PSCs recorded from MNs (control: 26.93±2.01pA; muscarine before 10min: 32.10±2.77pA; muscarine 15-30min: 25.35±2.31pA; washout: 26.10±2.41pA; n=12; $p<0.05$ repeated measures ANOVA with Tukey's post-test).

Muscarinic changes in synaptic activity could reflect decreased output from presynaptic neurons or direct modulation of synaptic transmission via pre- and/or

postsynaptic mechanisms. To address this, evoked activity was blocked with TTX and mPSCs were recorded in the presence of muscarine. Muscarine increased the inter-event interval (TTX: 551.04±93.13ms; TTX and muscarine: 751.37±122.76ms; washout (TTX): 720.36±89.67ms; n=15; $p<0.05$ Friedman's test with Dunn's post-hoc) and decreased the amplitude (TTX: 15.85±0.77pA; TTX and muscarine: 13.90±0.82pA; washout TTX: 13.04±0.66pA; n=15; $p<0.05$ repeated measures ANOVA with Tukey's post-test) of recorded mPSCs (figure 4.14b).

These results indicate that muscarine can have 2 distinct, time-dependent actions on synaptic inputs to MNs: an initial increase followed by a subsequent decrease in synaptic drive. The changes in both the amplitude and inter-event interval of mPSCs indicate that muscarinic modulation of synaptic transmission might involve activation of both pre- and postsynaptic receptors.

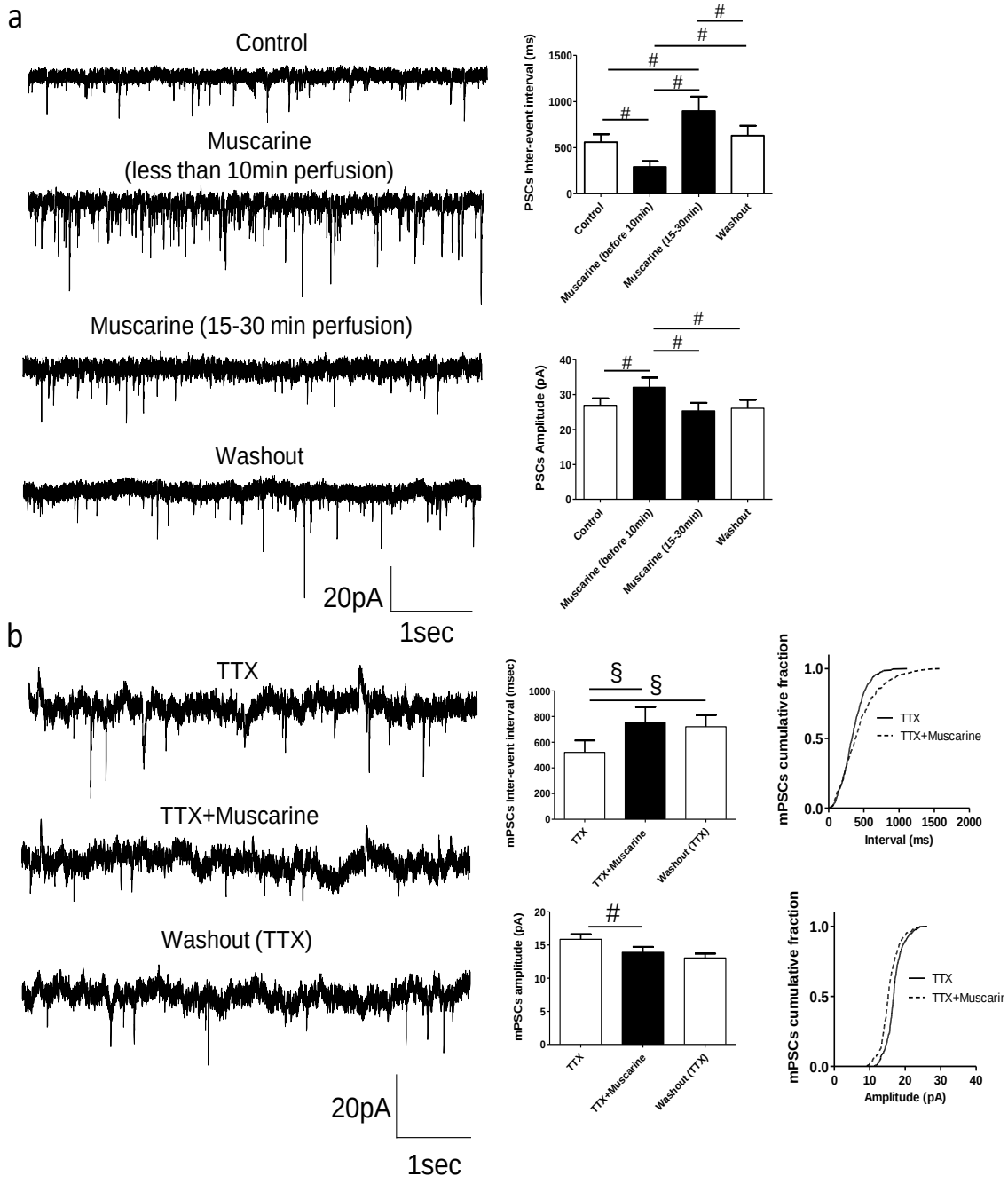


Figure 4.14 - Muscarine has a biphasic action on synaptic drive to MNs. **(a)** representative traces of recorded PSCs (left) with respective inter-event interval and amplitude histogram (right) illustrating the effects of muscarine (10µM) on PSCs (n=12); **(b)** example of acquired mPSCs (left) with inter-event interval and amplitude histogram (middle) and inter-event and amplitude cumulative fraction plots (right) of the effects of muscarine on spontaneous activity (n=15); all recordings were performed at a holding potential of -60mV. #$p<0.05$ repeated measures ANOVA with Tukey's post-test; §$p<0.05$ Friedman's test with Dunn's post-hoc

Having addressed the role of the general agonist, muscarine, on synaptic drive to MNs, the next step was to understand which receptor subtype, M2 or M3, was responsible for the actions of muscarine.

The potential role of M3 receptors in the time-dependent effects of exogenous activation of muscarinic receptors on synaptic inputs to MNs (see figure 4.14) was first assessed. The effects of muscarine, applied in the presence of the M2 antagonist to isolate actions mediated by M3 receptors, were assessed at multiple time points after drug application. When co-perfused with methoctramine, muscarine decreased PSCs inter-event interval (methoctramine: 436.18±99.64ms; methoctramine and muscarine before 10min: 212.09±48.18ms; methoctramine and muscarine 15-30min: 208.33±53.94ms; washout methoctramine: 522.33±95.66ms; n=12; Friedman's test with Dunn's post-hoc) but had no effect on PSC amplitude (methoctramine: 22.25±2.87pA; methoctramine and muscarine before 10min: 22.20±3.23pA; methoctramine and muscarine 15-30min: 22.67±3.09pA; washout methoctramine: 20.95±2.68pA; n=12) (figure 4.15a).

As discussed earlier, muscarinic receptor activation might involve both pre- and postsynaptic transduction mechanisms. This was again addressed for M3 receptor-dependent effects by investigating mPSCs in the presence of TTX. Muscarine co-applied with methoctramine in the presence of TTX (figure 4.15b) significantly increased mPSC inter-event interval (TTX and methoctramine: 611.00±73.03ms; TTX, methoctramine and muscarine: 947.45±156.59ms; washout TTX and methoctramine: 783.79±130.01ms; n=16; $p<0.05$ Friedman's test with Dunn's post-hoc) but did not affect mPSC amplitude (TTX and methoctramine: 14.98±1.09pA; TTX, methoctramine

and muscarine: 14.30±0.61pA; washout TTX and methoctramine: 13.38±0.82pA; n=16).

Activation of muscarinic receptors in the presence of methoctramine had an excitatory effect on the frequency of mPSCs, indicating that M3 receptors are involved in presynaptic modulation of last-order synapses on MNs. In addition, M2 muscarinic receptor blockade removed the delayed muscarine-induced decrease in synaptic inputs to MNs, suggesting that activation of M2 receptors decreases the neuronal drive to MNs.

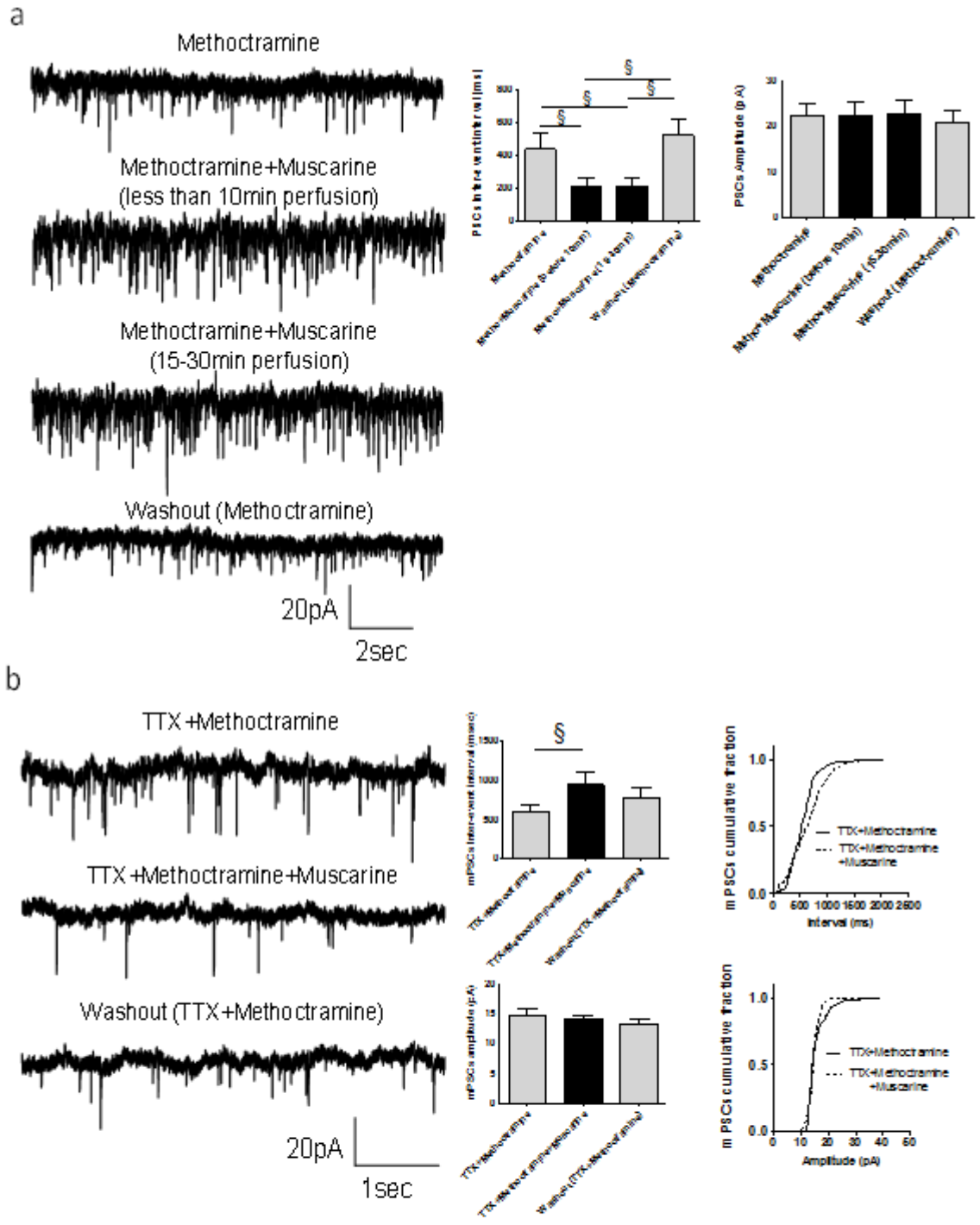


Figure 4.15 – Muscarine in the presence of M2 receptor blockade increases synaptic inputs to MNs; **(a)** PSCs recorded from a MN illustrating the effect on synaptic drive by muscarine (10μM) in the presence of methoctramine (10μM, n=12); **(b)** recorded mPSCs in the presence of methoctramine and muscarine (left) with respective inter-event interval and amplitude histogram (middle) and cumulative inter-event interval and amplitude fraction plots (right) (n=16); all recordings were performed at a holding voltage of -60mV; §$p<0.05$ Friedman's test with Dunn's post-hoc

To further study the role of M2 muscarinic receptor activation in the time-dependant muscarinic modulation of synaptic inputs to MNs, the non-selective agonist muscarine was perfused in the presence of the M3 receptor blocker 4-DAMP. Muscarine in the presence of 4-DAMP increased PSC inter-event interval (4-DAMP: 348.90±59.23ms; 4-DAMP and muscarine before 10min: 504.44±78.86ms; DAMP and muscarine 15-30min: 573.38±95.98ms; washout 4-DAMP: 468.10±77.45ms; n=10; $p<0.05$ repeated measures ANOVA with Tukey's post-test) and decreased PSC amplitude (4-DAMP: 17.08±1.42pA; 4-DAMP and muscarine before 10min: 15.65±1.28pA; 4-DAMP and muscarine 15-30min: 14.93±1.21pA; washout 4-DAMP: 14.52±1.42pA; n=10; $p<0.05$ repeated measures ANOVA with Tukey's post-test) (figure 4.16a).

Since activation of muscarinic receptors involves last-order synapse modulation, mPSCs were again also studied. The muscarine-induced decrease in mPSC amplitude remained in the presence of 4-DAMP (TTX and 4-DAMP: 13.94±1.04pA; TTX, 4-DAMP and muscarine: 12.79±0.7pA; Washout TTX and 4-DAMP: 11.92±0.76pA; n=16; $p<0.05$ repeated measures ANOVA with Tukey's post-test). However, muscarine had no effect on mPSC inter-event interval in the presence of 4-DAMP (TTX and 4-DAMP: 424.43±59.10ms; TTX, 4-DAMP and muscarine: 503.09±76.73ms; Washout TTX and 4-DAMP: 581.49±109.19ms; n=16) (figure 4.16b).

During M3 receptor blockade muscarine reduced synaptic drive to MNs indicating that M2 receptor activation decreases synaptic drive. Interestingly, M2 and M3 receptor activation have opposing effects on the synaptic drive to MNs. M3 receptors increase synaptic inputs whereas M2 receptors decrease the frequency of inputs to MNs. Analysis of mPSCs indicates that both M2 and M3 receptors directly

modulate synaptic connections with MNs, with M3 receptors likely located on presynaptic terminals and M2 receptors positioned postsynaptically.

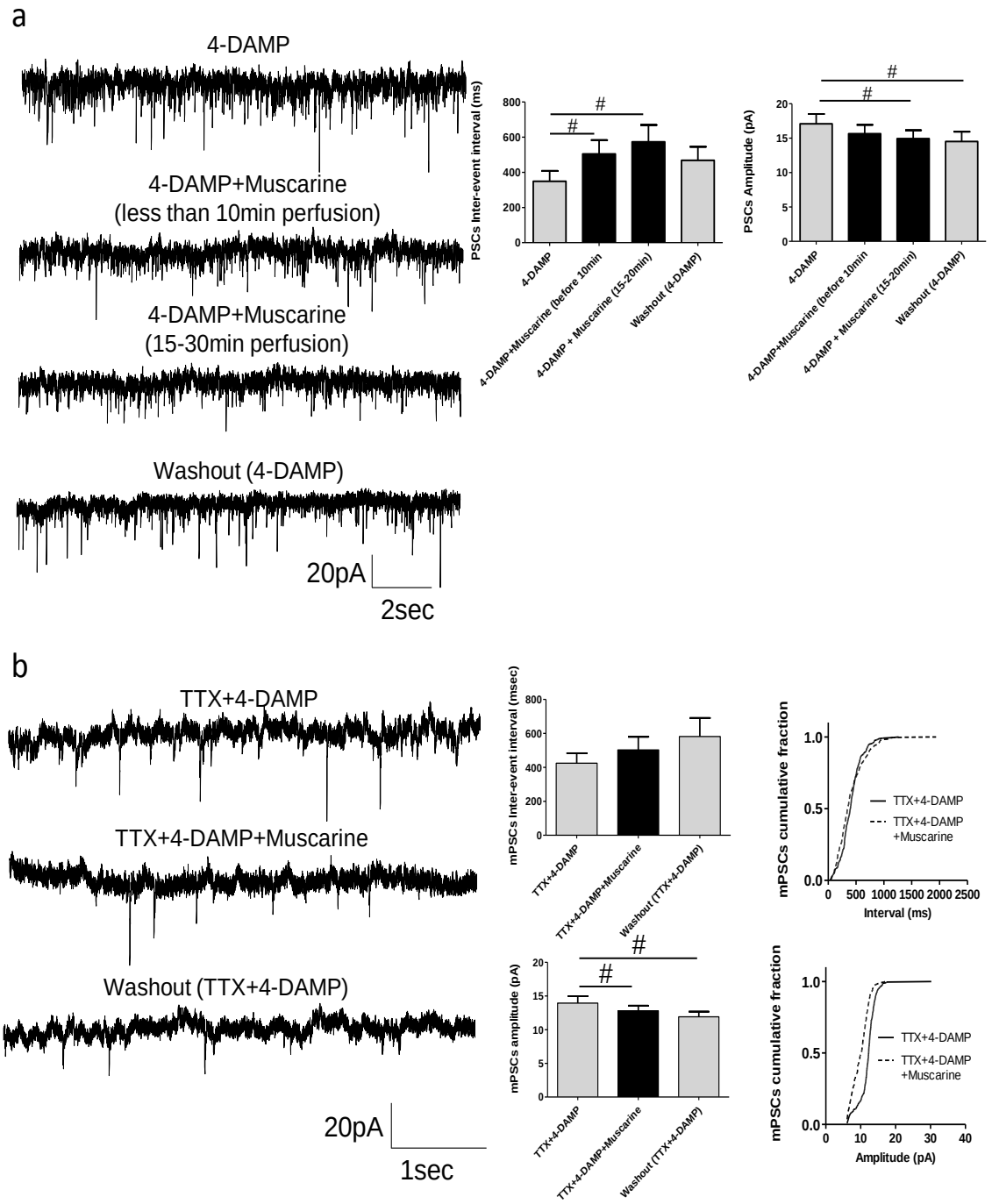


Figure 4.16 – Muscarine in the presence of 4-DAMP decreases synaptic inputs to MNs. **(a)** example of acquired PSCs (left) with inter-event interval and amplitude histogram graph (right) in the presence of 4-DAMP (2µM) co-perfused with muscarine (10µM, n=10); **(b)** representative traces of mPSCs (with 0.5µM TTX) recorded in the presence of 4-DAMP and muscarine (left) with average inter-event interval and amplitude plots (middle) and cumulative inter-event interval and amplitude fractions (right) (n=16). All

recordings were performed at a holding voltage of -60mV; #$p<0.05$ repeated measures ANOVA with Tukey's post-test

Results from previous sections indicated the presence of endogenous M2 and M3 receptor-dependent actions on MNs, even in spinal cord slice preparations (see figure 4.8). To address if endogenous release of ACh also modulates synaptic inputs to MNs via activation of muscarinic receptors, M2 and M3 receptor antagonists were perfused and PSCs were recorded from MNs. Perfusion of methoctramine in spinal cord slices (figure 4.17a) decreased PSC inter-event interval (control: 519.99±113.58ms; methoctramine: 375.10±96.49ms; washout: 578.15±114.24ms; n=8; repeated measures ANOVA with Tukey's post-test) but had no effect on PSC amplitude (control: 19.31±3.06pA; methoctramine: 17.93±2.91pA; washout: 15.82±1.44pA; n=8). Application of the M3 receptor antagonist 4-DAMP (figure 4.17b) increased PSC inter-event interval (control: 400.08±82.19ms; 4-DAMP: 630.86±106.93ms; washout: 735.02±133.45ms; n=6; $p<0.05$ repeated measures ANOVA with Tukey's post-test) but had no effect on PSC amplitude (control: 19.87±2.30pA; 4-DAMP: 18.13±2.24pA; washout: 16.72±2.02pA; n=6).

These data demonstrate that endogenous activation of M2 and M3 receptors in spinal cord slice preparations modulates synaptic drive to MNs: M2 receptors decrease synaptic drive whereas M3 receptors increase synaptic inputs to MNs.

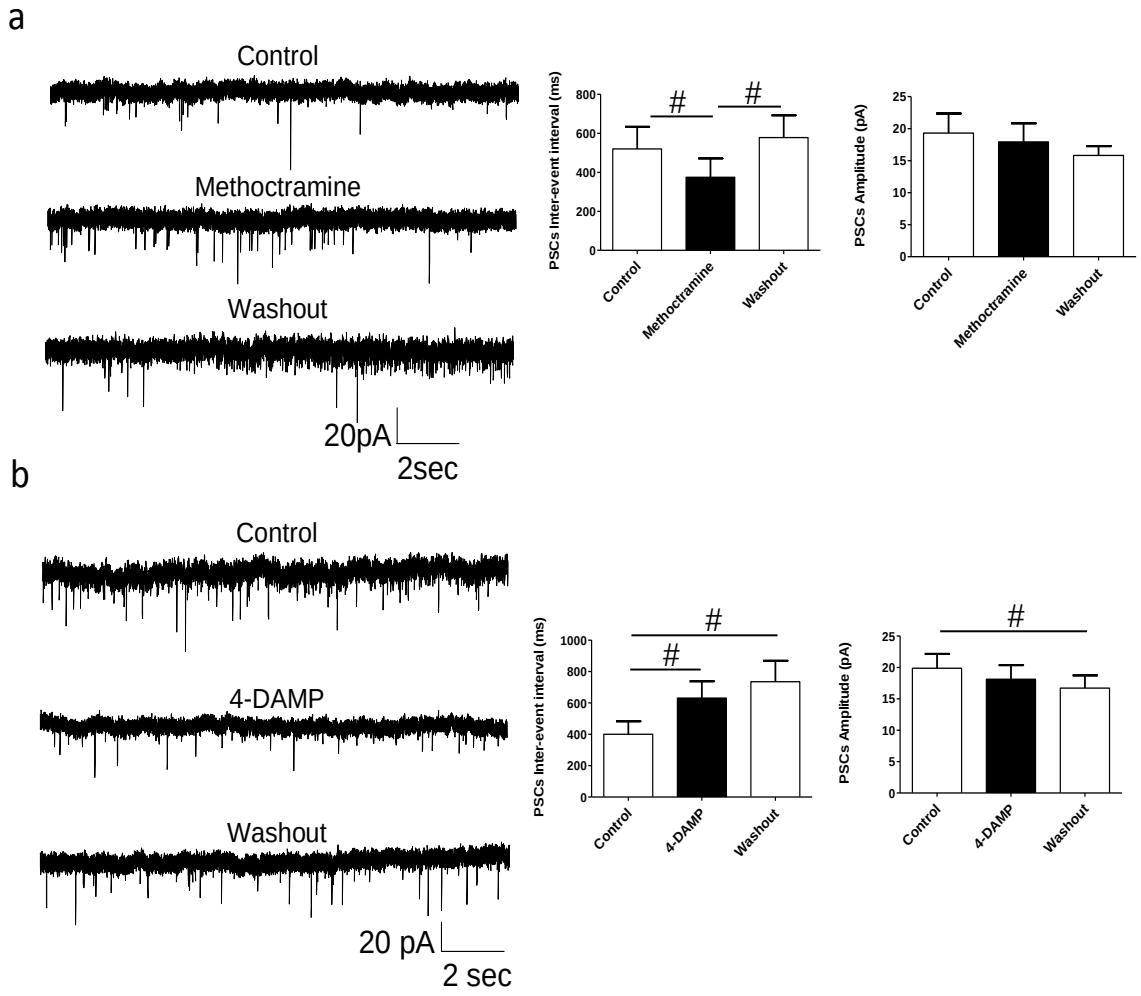


Figure 4.17 – M2 receptor antagonism increases and M3 receptor blockade decreases synaptic inputs to MNs. **(a)** recorded PSCs (left) with inter-event interval and amplitude histogram (right) in the presence of methoctramine (10µM, n=8); **(b)** example of acquired PSCs (left) with inter-event interval and amplitude histogram graph (right) in the presence of 4-DAMP (2µM, n=6); All recordings were performed at a holding voltage of -60mV; #p<0.05 repeated measures ANOVA with Tukey's post-test

4.2. Chemogenetic interrogation of Pitx2^{+} IN-derived cholinergic modulation of MNs and locomotor output

Having characterized muscarinic modulation of MN function and lumbar spinal locomotor circuitry, this work next focused on understanding which subpopulations of cholinergic INs are responsible for some of the endogenous, muscarinic receptor-mediated effects that were revealed via pharmacological experiments. Pitx2^{+} INs are the only group of cholinergic spinal INs that have been genetically identified (Zagoraiou *et al.*, 2009). These INs form large C-bouton synapses on MNs with indirect evidence suggesting that Pitx2^{+} cells modulate MN output through postsynaptic M2 receptors (Miles *et al.*, 2007; Zagoraiou *et al.*, 2009; Witts *et al.*, 2014). Given the previously indirect methods used to investigate the likely roles of Pitx2^{+} INs and C-boutons in controlling MN output, the present study sort to provide more direct evidence of the effect of Pitx2^{+} cell activation on MN properties.

DREADD technology allows the expression of excitatory (G_q) or inhibitory (G_i) metabotropic receptors that can only be activated with a designer drug (CNO) thus allowing chemogenetic activation or inhibition of neuronal activity (Zhu *et al.*, 2017). Meanwhile, the cre-lox recombination system enables the selective expression of genes in specific neuronal subtypes. DREADD technology and cre-lox recombination were therefore used to enable the selective manipulation of Pitx2^{+} INs in order to help understand the cholinergic mechanisms by which this particular subset of INs modulates motor output.

DREADD mice were used to test the role of the activation (Pitx2-Cre;CHRM3) and inhibition (Pitx2-Cre;CHRM4) of Pitx2^{+} INs on MN function and locomotor network output. Considering that the majority of Pitx2^{+} INs are spontaneously active at

low firing rates (Zagoraiou *et al.*, 2009), it was first assessed whether DREADD-engineered proteins could increase or decrease tonic Pitx2^{+} IN activity in spinal cord slices from Pitx2-Cre;TdTom;CHRM3/CHRM4 mice (sections 4.2.1 and 4.2.4). After verifying the ability of DREADDs to manipulate Pitx2^{+} IN activity, experiments investigated whether MN output was altered in response to activation of Pitx2^{+} INs with CNO in intact spinal cords from Pitx2-Cre;CHRM3 mice. Considering the indirect evidence suggesting that M2 receptors are responsible for Pitx2^{+} IN-mediated modulation of MN output (Miles *et al.*, 2007; Witts *et al.*, 2014) and that Kv2.1 channels are clustered postsynaptically at C-bouton synapses (Wilson *et al.*, 2004; Deardorff *et al.*, 2014), it was explored if these proteins were part of the mechanism underlying Pitx2^{+} IN-mediated modulation of MN function (sections 4.2.1-4.2.3). After investigating the mechanism through which this subset of cholinergic INs affects MN output, the next step was to investigate the role of Pitx2^{+} IN-derived cholinergic modulation in rhythmically active locomotor networks. Since Pitx2^{+} INs are active during fictive locomotion, with their activity typically in phase with the ventral root output of the lumbar segment in which they reside (Zagoraiou *et al.*, 2009), inhibition of Pitx2^{+} IN activity in Pitx2-Cre;CHRM4 mice was utilised to investigate their contribution to drug-induced locomotor-related output. This was assessed via recordings from single MNs or ventral roots of isolated spinal cord preparations (section 4.2.1-4.2.7).

The effects of the designer drug CNO and its metabolites have not been completely characterized however the majority of the authors suggest that it is pharmacologically inert at lower doses and concentrations (Roth, 2016). To insure that the effects of CNO application in tissue in which DREADDS were selectively

expressed by Pitx2$^+$ INs was due to their chemogenetic activation and inactivation, CNO was perfused in spinal cords from animals that had the LoxP-STOP-LoxP cassette for DREADDs but did not express Cre-recombinase rendering the engineered receptor expression null (sections 4.2.1, 4.2.2 and 4.2.6).

4.2.1. Pitx2$^+$ INs can be excited using DREADD technology

It was first tested whether Pitx2$^+$ INs could be activated using excitatory (hM3Dq receptor) DREADD expression. This was assessed using whole-cell patch-clamp recordings of Pitx2$^+$ INs in spinal cord slices obtained from Pitx2-Cre;TdTomato;CHRM3 mice. In these mice Pitx2$^+$ cells express both the hM3Dq receptor and the TdTomato red fluorescent reporter, which allowed them to be identified for single-cell electrophysiology. As reported previously (Zagoraiou et al., 2009), Pitx2$^+$ INs were found to be tonically active at rest (figure 4.18a). Application of CNO (1µM) was found to increase the tonic firing rate of Pitx2$^+$ INs in slices from Pitx2-Cre;TdTomato;CHRM3 mice (control: 1.82±0.48Hz; CNO: 2.98±0.84Hz; n=8; $p<0.05$ paired *t*-test).

The exact mechanism of action of CNO on DREADD receptors has often been a theme of controversy with recent work suggesting that the by-product of CNO – clozapine – is the compound responsible for activation of the engineered receptors (Gomez *et al.*, 2017). Authors have pointed out that there might be nonspecific actions of CNO and clozapine both *in vivo* and *in vitro* (MacLaren *et al.*, 2016; Roth, 2016) implying the need for rigorous control experiments. To address this, CNO was perfused in spinal cord slices from Pitx2-Cre;TdTomato neonates that express the red fluorescent reporter in Pitx2$^+$ INs, but not the DREADD receptor. Changes in spontaneous firing

activity were recorded to check if CNO would significantly affect the output of these INs. In spinal cord slices from Pitx2-Cre;TdTomato mice application of CNO did not change spontaneous firing (control: 0.921±0.094Hz; CNO: 0.851±0.103Hz; n=5 (figure 4.18b).

Taken together, these data confirm that Cre recombinase-based selective expression of the hM3Dq receptor, can be used to selectively activate Pitx2^{+} INs.

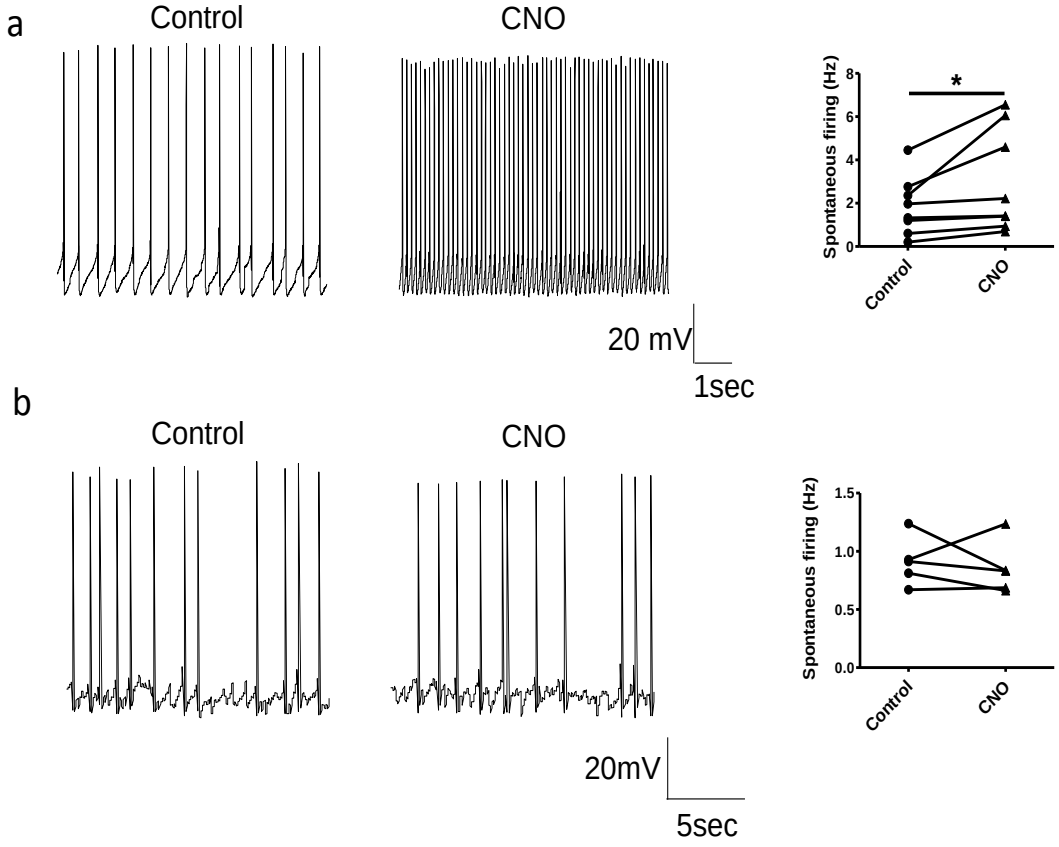


Figure 4.18 – Pitx2^{+} INs can be activated using DREADD technology. **(a)** firing of a Pitx2^{+} IN from a Pitx2-Cre;TdTom;CHRM3 mouse in the presence of CNO (1µM, left) with mean spontaneous firing plot (right, n=8); **(b)** Spontaneous firing of a Pitx2^{+} IN from Pitx2-Cre;TdTom mice before and in the presence of CNO (left) and respective firing plot (right, n=5); *p<0.05 paired *t*-test

4.2.2. Chemogenetic activation of Pitx2$^+$ INs induces an inward current in MNs

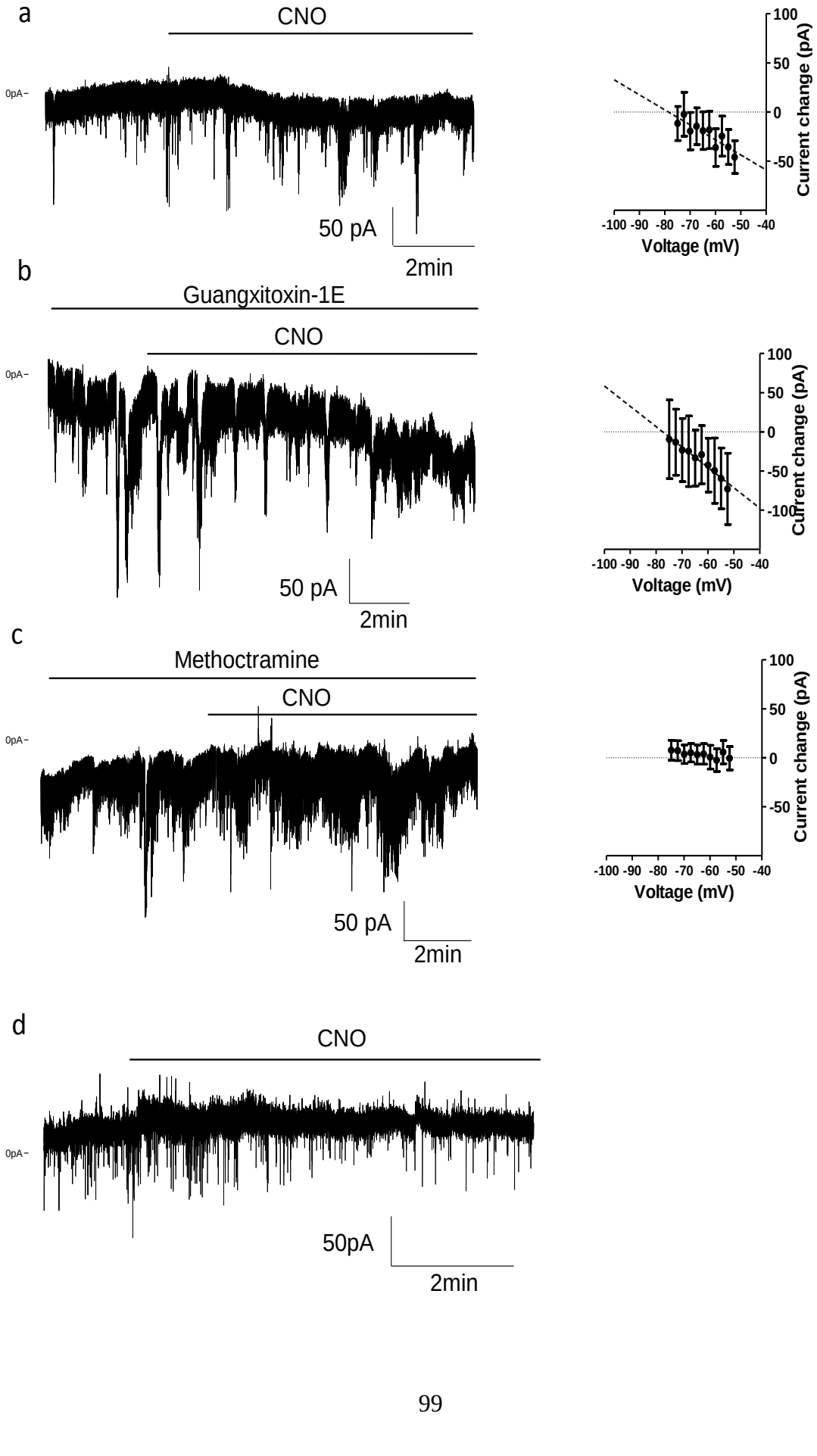
Knowing that Pitx2$^+$ INs could be activated by CNO in Pitx2;CHRM3 mice, the effects of Pitx2$^+$ INs and their C-bouton contacts with MNs were next assessed by performing whole-cell patch-clamp recordings of MNs in whole spinal cord preparations. It was first investigated whether Pitx2$^+$ IN activation induced any subthreshold currents in MNs.

In spinal cords from Pitx2;CHRM3 mice, activation of Pitx2$^+$ INs with CNO elicited an inward current in MNs (-37.49±5.88pA, n=14) that was associated with an increase in input resistance (control: 76.82±8.68MΩ, CNO: 89.69±11.46MΩ, reversal -78.57mV, n=11; figure 4.19a). Given that M2 receptors are clustered postsynaptically at C-bouton synapses formed by Pitx2$^+$ INs on MNs, it was then tested whether this inward current was blocked by methoctramine. In the presence of methoctramine, the application of CNO no longer induced an inward current in MNs (-2.16±4.07pA, n=9; figure 4.19b). It was next assessed whether the blockade of Kv2.1 channels, which are also clustered postsynaptically at C-bouton synapses, might underlie this change in holding current and associated increase in input resistance in MNs upon Pitx2$^+$ IN activation. However, when CNO was applied in the presence of the Kv2.1 blocker guangxitoxin-1E (50nM), inwards currents (-29.50±6.51pA, n=10) and an increase in input resistance (guangxitoxin-1E: 50.09±3.77MΩ, guangxitoxin-1E and CNO: 56.74±3.62MΩ, reversal -77.49mV, n=8) were still recorded in MNs (figures 4.19c).

In CHRM3 mice the absence of Cre-recombinase means the CHRM3 gene is not expressed. In these animals CNO was perfused and electrophysiological properties were recorded from MNs from intact spinal cords to check for any nonspecific effects on

MNs when hM3Dq was not expressed in Pitx2^{+} INs. As seen in figures 4.19d, in CHRM3 mice, CNO did not induce change in holding current in MNs (n=14).

These data demonstrate that activation of Pitx2^{+} INs leads to a depolarizing current and increase in input resistance in MNs which involves activation of M2 muscarinic receptors but does not involve Kv2.1 channels located at the C-bouton synapse.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CNO
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-100 -90 -80 -70 -60 -50 -40
Voltage (mV)
Current change (pA)
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Guangxitoxin-1E
CNO
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50 pA
2min
100
50
0
-50
-100
-100 -90 -80 -70 -60 -50 -40
Voltage (mV)
Current change (pA)
c
Methoctramine
CNO
0pA
50 pA
2min
100
50
0
-50
-100 -90 -80 -70 -60 -50 -40
Voltage (mV)
Current change (pA)
d
CNO
0pA
50pA
2min

Figure 4.19 – Pitx2$^+$ INs activation induces an inward current in MNs through the activation of M2 muscarinic receptors. voltage clamp currents (left) and current-voltage relation (right) from MNs in intact spinal cord preparations from Pitx2-Cre;CHRM3 mice in the presence of **(a)** CNO (1µM, n=14 and n=11) and **(b)** CNO co-applied with methoctramine (10µM, n=9 and n=8) or **(c)** guangxitoxin-1E (50nM, n=10 and n=8). **(d)** example of changes in voltage-clamp current from a MN from CHRM3 mice (n=14).

4.2.3. Chemogenetic activation of Pitx2$^+$ INs increases MN firing via activation of M2 receptors and regulation of Kv2.1 channels

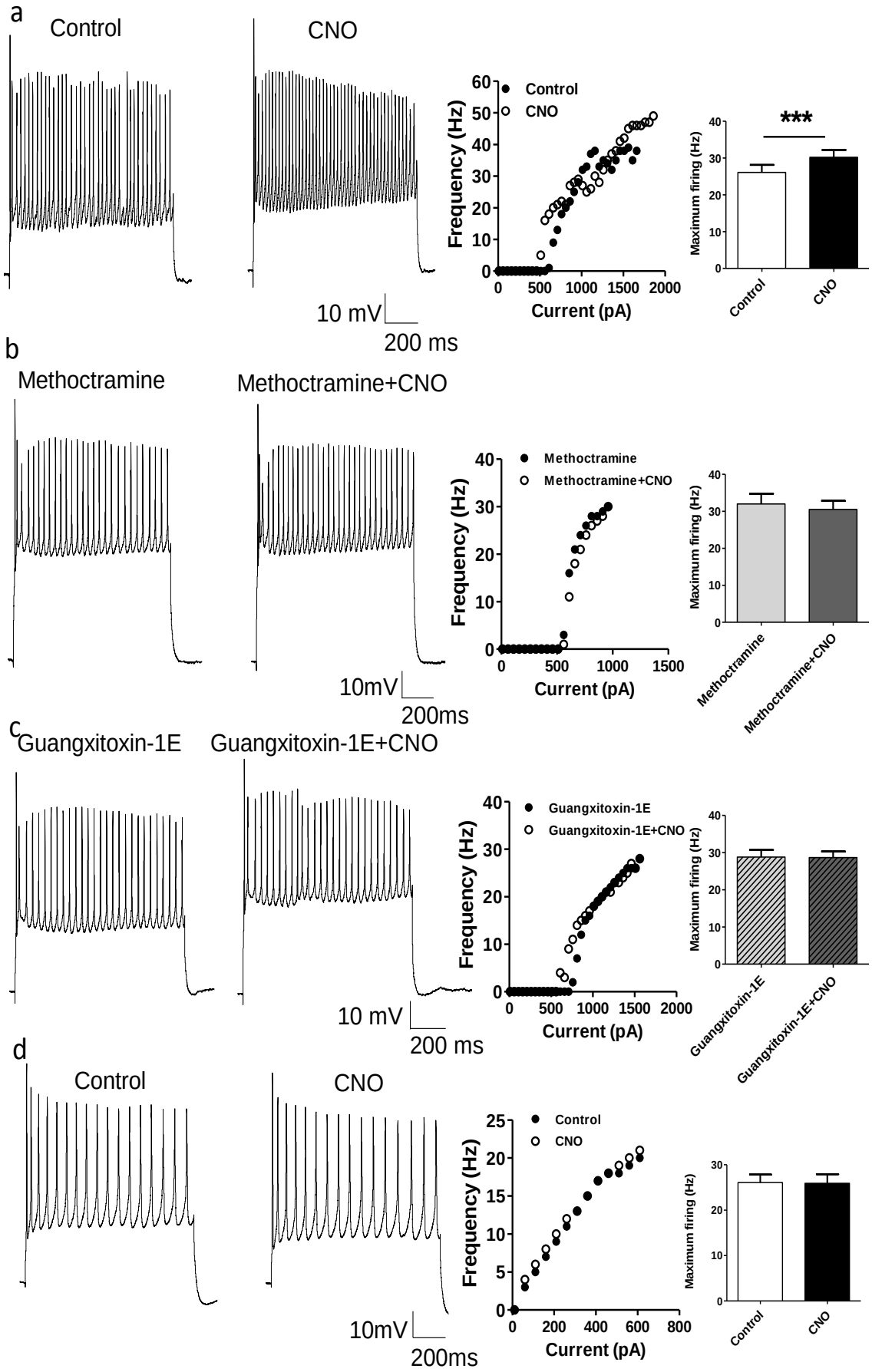
Following the demonstration that Pitx2$^+$ INs could be activated with CNO in Pitx2;CHRM3 mice and that this resulted in modulation of the holding current and input resistance of MNs, it was next assessed whether activation of Pitx2$^+$ INs modulated the firing output of MNs.

MN input-output relationships were investigated using a series of depolarising current steps (50pA increments; 1s duration) applied in current-clamp mode during whole-cell patch-clamp recordings of MNs in isolated spinal cord preparations. As seen in figure 4.20a, when Pitx2$^+$ INs were activated using CNO, the maximum firing frequency of MNs increased (control: 26.08±2.05Hz; CNO: 30.20±2.00Hz; n=25; $p<0.001$ paired t-test).

Next, the postsynaptic receptors and channels involved in this Pitx2$^+$ IN-mediated modulation in MN output were assessed. Activation of Pitx2$^+$ INs in the presence of the M2 receptor antagonist methoctramine had no effect on MN firing (methoctramine: 32.00±2.72Hz; methoctramine and CNO: 30.50±2.33Hz; n=12). Similarly, blockade of Kv2.1 channels with guangxitoxin-1E (50nM) prevented changes in MN firing upon Pitx2$^+$ IN activation (guangxitoxin-1E: 28.80±1.90Hz; guangxitoxin-1E and CNO: 28.67±1.66Hz; n=15) (figures 4.20b-c).

In CHRM3 only animals lacking DREADD expression, CNO did not affect maximum firing (control: 25.92±2.00Hz; CNO: 26.33±1.68Hz; n=17; figure 4.20d). These results again demonstrate that CNO alone does not affect MN function in mice.

These results indicate that activation of $Pitx2^{+}$ INs through the hM3Dq receptor signalling pathway resulted in activation of M2 muscarinic receptors juxtaposed to C-boutons, which led to an increase in the maximum firing capability of MNs. In addition, data support that Kv2.1 channels are required for this increase in MN output.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Frequency (Hz)
Current (pA)
Maximum firing (Hz)
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Methoctramine+CNO
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Guangxitoxin-1E
Guangxitoxin-1E+CNO
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Guangxitoxin-1E+CNO
Frequency (Hz)
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Maximum firing (Hz)
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Guangxitoxin-1E+CNO
d
Control
CNO
10mV
200ms
Control
CNO
Frequency (Hz)
Current (pA)
Maximum firing (Hz)
Control
CNO

Figure 4.20 – Pitx2$^+$ INs increase MN maximum firing through the activation of M2 muscarinic receptors and subsequent modulation of Kv2.1. Representative firing step (left) with respective current-frequency plot (middle) and histogram of MN maximum firing averages (right) from Pitx2-Cre;CHRM3 mice showing that **(a)** activation of Pitx2$^+$ INs (CNO 1µM) increased the maximum firing rate observed in MNs (n=25), which was **(b)** blocked by methoctramine (10µM, n=12) and **(c)** guangxitoxin-1E (50nM, n=15); **(d)** example of firing step (left) with respective frequency-current plot (middle) and averaged maximum firing (n=17, right) from CHRM3 mice MNs in presence of CNO.*p<0.001 paired *t*-test

4.2.4. Pitx2$^+$ INs increase MN firing via M2 receptor/Kv2.1 channel-dependent shortening of action potential half-width

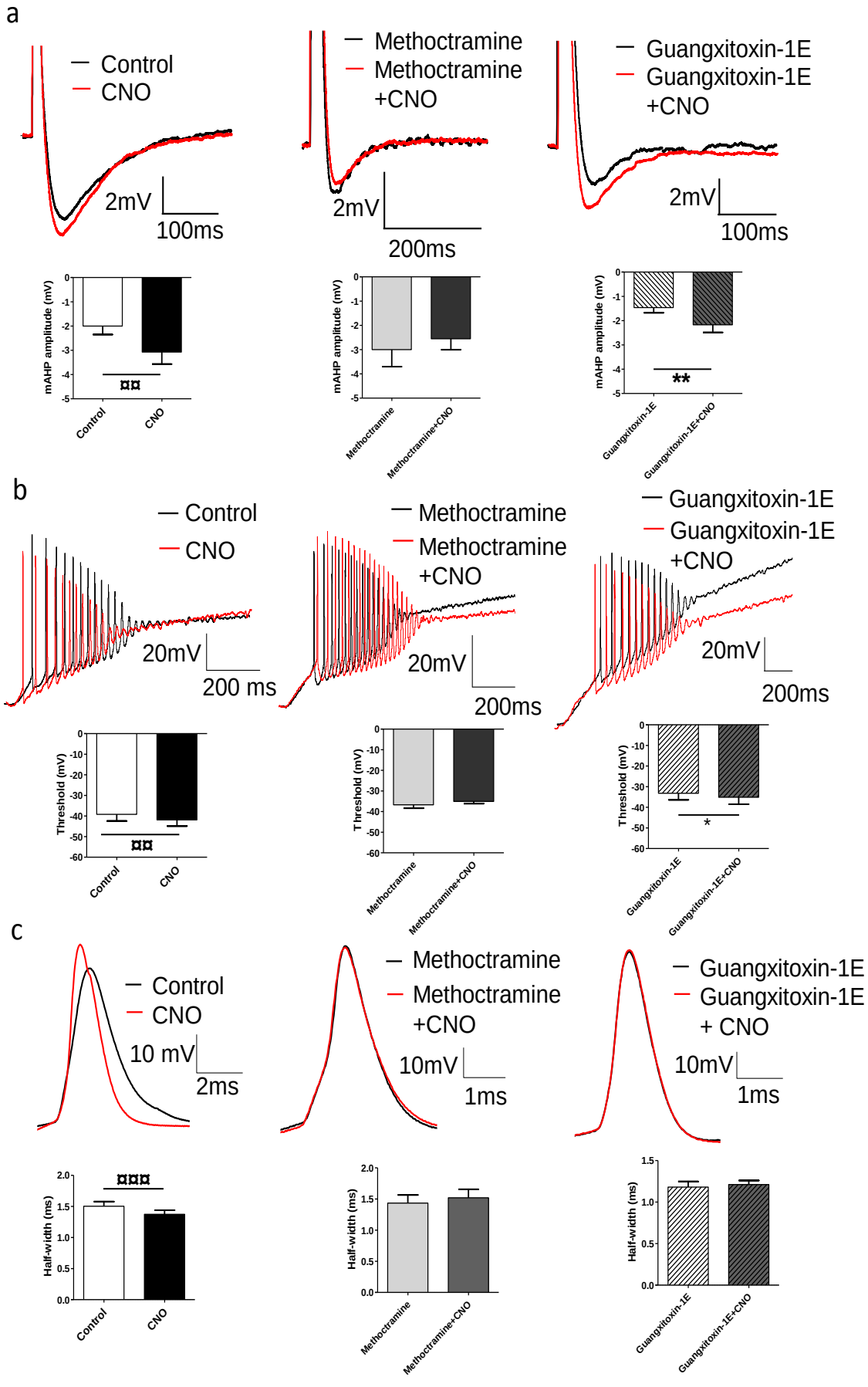
The mechanisms by which activation of Pitx2$^+$ INs and their C-bouton contact with MNs modulates MN output was next investigated. Previous, indirect evidence suggested that activation of M2 receptors at C-boutons is likely to increase MN output through a reduction in the amplitude of the mAHP (Miles *et al.*, 2007). It was therefore first assessed whether chemogenetic activation of Pitx2$^+$ INs reduced the action potential AHP of MNs within isolated spinal cord preparations from Pitx2-Cre;CHRM3 mice. Single action potentials were evoked in current-clamp mode using brief (10ms) depolarising current steps and mAHPs were measured. Surprisingly, DREADD-mediated activation of Pitx2$^+$ INs increased the mAHP amplitude in MNs (control: -2.00±0.35mV; CNO: -3.07±0.50mV; n=22; p<0.01 Wilcoxon matched pairs test). This effect was dependent on M2 receptor activation, as revealed by its block by methoctramine (methoctramine: -3.00±0.70mV; methoctramine and CNO: -2.55±0.45mV; n=7), but did not involve Kv2.1 channels, as indicated by a lack of blockade by guangxitoxin-1E (guangxitoxin-1E: -1.46±0.22mV; guangxitoxin-1E and CNO: -2.17±0.32mV; n=11; p<0.05 paired *t*-test) (figure 4.21a).

The increase in MN mAHP observed after activation of Pitx2$^+$ INs indicates that mAHP modulation does not underlie increases in MN firing, therefore other protocols were used to further explore potential mechanisms by which Pitx2$^+$ INs increase MN output. A depolarising current ramp (1s long) was used to check for changes in MN action potential threshold. As illustrated in figure 4.21b, Pitx2$^+$ INs activation decreased action potential threshold (control: -39.20±3.20mV; CNO: -41.85±3.03mV; n=12; $p<0.05$ Wilcoxon matched pairs test), even in the presence of the Kv2.1 blocker (guangxitoxin-1E: -33.17±3.20mV; guangxitoxin-1E and CNO: -35.12±3.40mV; n=5; $p<0.05$ paired *t*-test), but not with prior M2 receptor blockade (methoctramine: -37.70±1.65mV; methoctramine and CNO: -35.70±1.04mV; n=5). Thus, although a Pitx2$^+$ IN-dependent decrease in firing threshold may contribute to increased MN excitability, given that guangxitoxin-1E did not block the change in firing threshold, this is unlikely to underlie increases in the maximum rate of MN firing which were blocked by the Kv2.1 channel antagonist (see figure 4.20).

Modulation of voltage-gated K^+ channels, such as Kv2.1 channels, can increase neuronal firing by decreasing the action potential half-width (Bean, 2007; Fletcher *et al.*, 2017). To address if the activation of Pitx2$^+$ INs affected action potential kinetics, the half-width of single action potentials recorded from MNs of Pitx2-Cre;CHRM3 mice was next investigated (figure 4.21c). Following activation of Pitx2$^+$ INs with CNO, the half-width of MN action potentials was found to decrease (control: 1.50±0.07ms; CNO: 1.37±0.07ms; n=20; $p<0.001$ Wilcoxon matched pairs test). This effect was blocked by both methoctramine (methoctramine: 1.44±0.13ms; methoctramine and CNO: 1.52±0.14ms; n=11) and guangxitoxin-1E (guangxitoxin-1E: 1.19±0.07ms; guangxitoxin-1E and CNO: 1.21±0.05ms; n=13), demonstrating a

dependence upon M2 receptors and Kv2.1 channels respectively. The kinetics of voltage-gated Na^+ channels can affect the action potential rise time but also the half-width of action potentials (Bean, 2007). The potential influence of modulation of Na^+ channels in $Pitx2^+$ IN-dependent reduction in MN action potential half-width was therefore also assessed by measuring action potential rise-time. However, no significant change in the rise time of MN action potentials was observed after activation of $Pitx2^+$ INs by CNO (control: 0.89±0.07ms; CNO: 0.85±0.05ms; n=20).

These results indicate that chemogenetic activation of $Pitx2^+$ INs results in an increase in MN output through a mechanism involving activation of M2 muscarinic receptors and a Kv2.1 channel-dependent shortening of MN spike half-width. The activation of $Pitx2^+$ INs also decreased voltage threshold and increased mAHP amplitude in MNs which was dependent on M2 receptors but not Kv2.1. This suggests that $Pitx2^+$ INs modulate MN output through multiple pathways that are dependent on M2 receptor signalling cascades at the C-bouton synapse, not all of which involve actions on Kv2.1 channels. Overall, these experiments provide novel evidence of direct modulation of MN output by $Pitx2^+$ INs and their C bouton synapses.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CNO
2mV
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Methoctramine
Methoctramine +CNO
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Guangxitoxin-1E
Guangxitoxin-1E+CNO

Figure 4.21 - Activation of Pitx2$^+$ INs increases MN output through a reduction of the spike half-width and involves M2 receptors and Kv2.1 channels. **(a)** truncated single action potentials illustrating changes in the amplitude of the mAHP after DREADD-based activation of Pitx2$^+$ INs (CNO 1µM, n=22), in the presence of the M2 antagonist (10µM, n=7) and guangxitoxin-1E (50nM, n=11) with respective average plots; **(b)** example of MN response to a depolarizing current ramp (1s duration) illustrating action potential threshold upon perfusion of CNO (n=12), methoctramine with CNO (n=5) and guangxitoxin-1E with CNO (n=5); **(c)** superimposed action potentials recorded from a MN before and after CNO illustrating action potential half-width following activation of Pitx2$^+$ INs (n=20), with methoctramine (n=11) and the Kv2.1 blocker (n=13); *p<0.05 paired *t*-test; ¤¤p<0.01 ¤¤¤p<0.001 Wilcoxon matched pairs test

4.2.5. Pitx2$^+$ INs can be inhibited using DREADD technology

Having characterized mechanisms of Pitx2$^+$ IN-mediated modulation of MN firing, the next experiments aimed to understand the role these neurons play in controlling behaviourally-relevant MN output, such as that produced to control locomotion. Pitx2$^+$ INs are known to be active during fictive locomotion (Zagoraiou *et al.*, 2009). Therefore, increasing their activity further with excitatory DREADDs may not clearly reveal their roles and could paradoxically reduce their activity due to a 'depolarising block' involving inactivation of Na^+ channels. A strategy utilising inhibitory DREADDs (hM4Di inhibitory receptor) was therefore chosen to help interrogate the role of Pitx2$^+$ INs in controlling locomotor-related MN output.

It was first assessed whether the hM4Di inhibitory receptor (encoded by the CHRM4 gene) could be used to selectively inhibit Pitx2$^+$ INs. Whole-cell patch-clamp recordings were performed from Pitx2$^+$ INs in slices prepared from Pitx2-Cre;TdTomato;CHRM4 mice, which selectively express both the fluorescent TdTomato protein and the inhibitory DREADD receptor in Pitx2$^+$ INs. As seen in figure 4.22 perfusion of CNO significantly decreased the spontaneous firing of Pitx2$^+$ INs (control: 1.64±0.43Hz; CNO: 1.17±0.39Hz; n=10; p<0.05 paired *t*-test). Thus, Pitx2$^+$ INs can be inhibited with DREADDs.

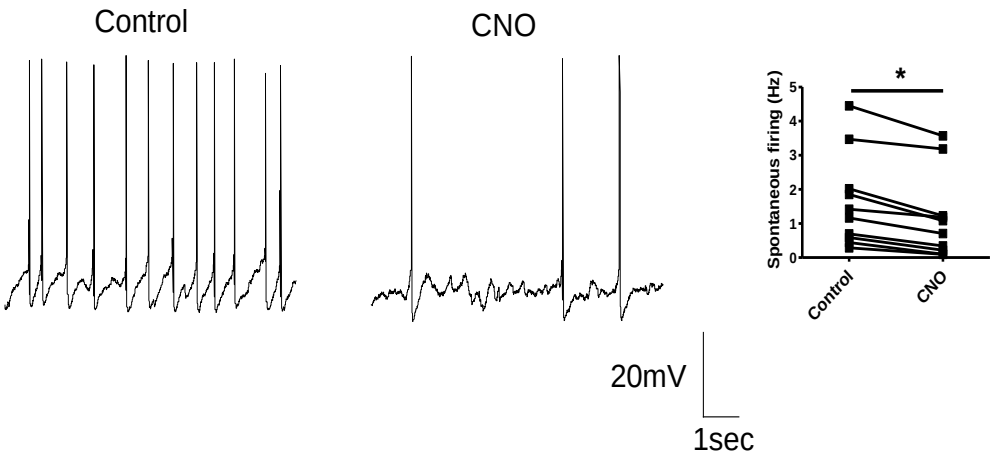


Figure 4.22 – Pitx2$^+$ INs activity can be decreased using DREADDs. Spontaneous firing of a Pitx2$^+$ IN from Pitx2-Cre;TdTom;CHRM4 mice before and in the presence of CNO (1µM) with mean spiking plot (n=10); *p<0.05 paired *t*-test

4.2.6. Chemogenetic inhibition of Pitx2$^+$ INs reveals tonic currents in MNs

Having established that DREADD technology can be used to inhibit Pitx2$^+$ IN activity, the effects of this inhibition on MN function were next investigated. Whole-cell patch-clamp recordings were performed from MNs within intact spinal cord preparations from Pitx2-Cre;CHRM4 mice. Given that the goal was to assess the effects of reducing Pitx2$^+$ IN activity, MN recordings were performed in the presence of drugs used to induce locomotor-related activity (NMDA, DA and 5-HT) because Pitx2$^+$ INs are known to be particularly active during fictive locomotion. It was first accessed whether inhibition of Pitx2$^+$ INs blocked tonic inwards currents expected to be induced by Pitx2$^+$ IN activity (see figure 4.19a). Inhibition of Pitx2$^+$ INs with CNO elicited a outward current (102.04±13.92pA, n=11) that was associated with a decrease in input resistance (NMDA, DA and 5-HT: 91.92±10.33MΩ, NMDA, DA, 5-HT and CNO: 65.57±5.17MΩ, reversal -114.60mV, n=6). This effect of CNO was blocked by methoctramine (n=3) (figure 4.23).

These results demonstrate that, in the presence of locomotor drugs, inhibition of Pitx2^{+} INs hyperpolarizes MNs and decreases their input resistance via an M2-dependent pathway. These effects are opposite to those observed using DREADD-based activation of Pitx2^{+} INs (Pitx2-Cre;CHRM3 mice; section 4.2.1). This indicates that inhibition of Pitx2^{+} INs blocks a resting depolarizing current that is present due to tonic activity of Pitx2^{+} INs.

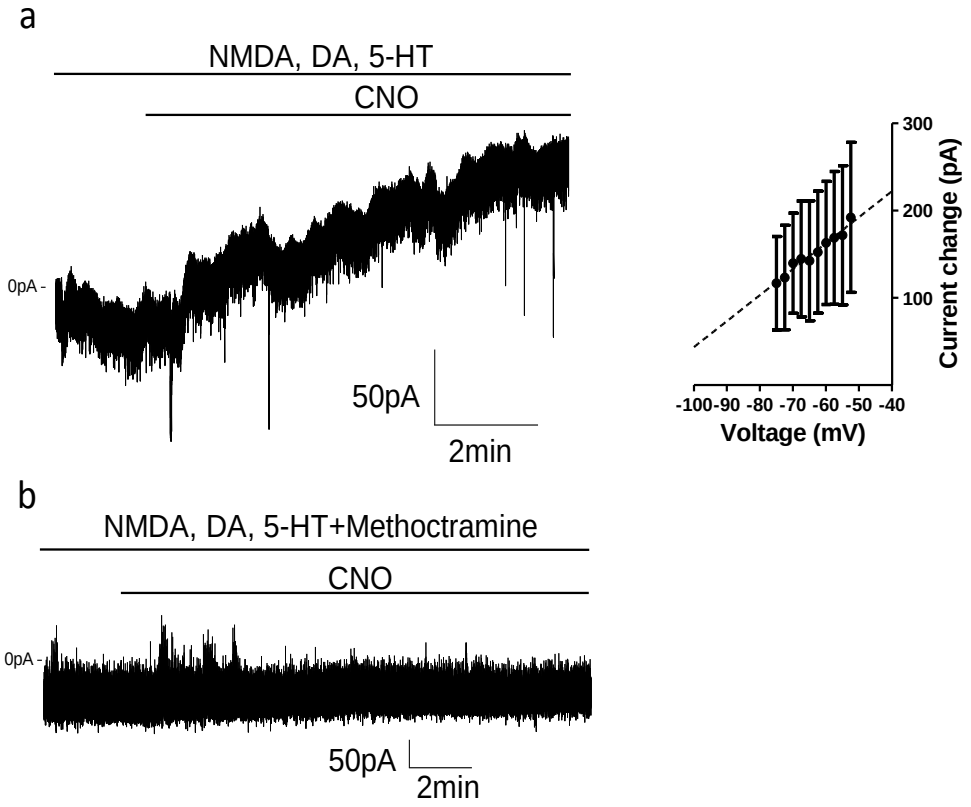


Figure 4.23 –Inhibition of active Pitx2^{+} INs elicits an outward during fictive locomotion. **(a)** MN recording in voltage clamp mode from intact spinal cord preparations illustrating changes in current (left, n=11) and respective current-voltage relation (right, n=6) after Pitx2^{+} IN activation (CNO 1µM) and **(b)** change in holding current in the presence of methoctramine (10µM, n=3) in Pitx2-Cre;CHRM4 mice.

4.2.7. Chemogenetic inhibition of Pitx2^{+} INs reduces MN firing

To address if DREADD-based inhibition of Pitx2^{+} INs would impact MN output, single-cell recordings were performed from MNs during drug-induced

locomotion. Passive, gap-free recordings were used to assess the firing rates of MNs that demonstrated locomotor-related bursting activity, while current steps were applied to MNs that were not active or exhibited very few action potentials during the pharmacologically-induced locomotor-related activity. Figures 4.24 shows that the rate of MN firing per burst during locomotor-related bursting was reduced when Pitx2^{+} INs were chemogenetically inhibited (control: 12.20±1.99Hz; CNO: 4.31±1.05Hz; n=4; $p<0.05$ paired *t*-test). Analysis of the firing rates of MNs in which current steps could be applied revealed a reduction in maximum firing rate when Pitx2^{+} INs were inhibited with CNO (control: 24.57±3.20Hz; CNO: 12.43±2.30Hz; n=7; $p<0.05$ paired *t*-test).

Together these results demonstrate that inhibition of Pitx2^{+} INs in Pitx2-Cre;CHRM4 mice reduces MN firing. These results support that Pitx2^{+} INs are important for the increase in firing output of MNs during episodes of fictive locomotion.

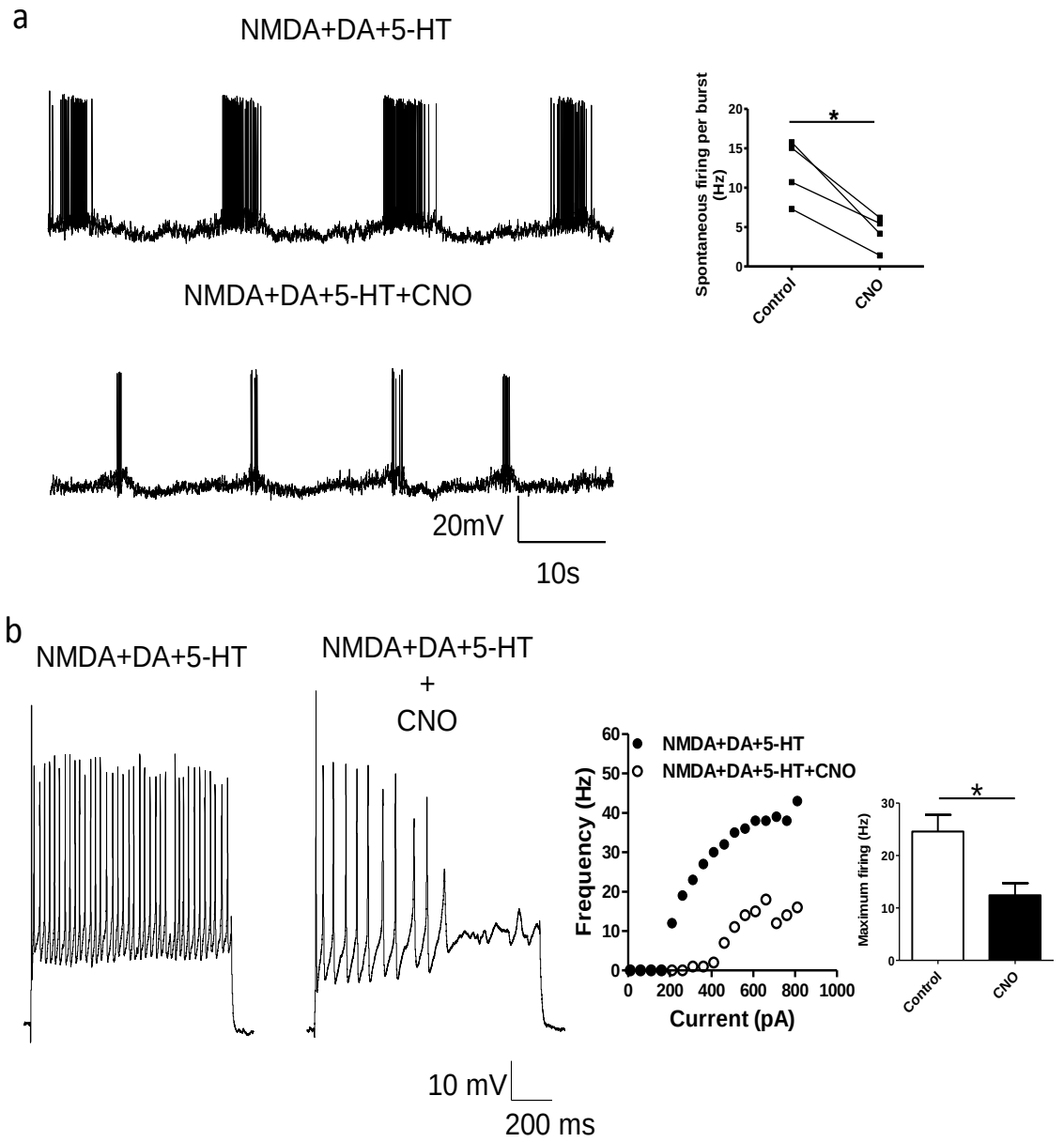


Figure 4.24 – DREADD-mediated inhibition of Pitx2^{+} INs decreased MN firing during episodes of fictive locomotion. **(a)** spontaneous firing from a MN during fictive locomotion (left) with average firing frequency per burst plots (right, n=4) and **(b)** representation of MN maximum output in response to increments of current injection after Pitx2^{+} INs inhibition (CNO 1µM, left) with respective current-frequency plot (middle) and firing averages histogram (right, n=7); *p<0.05 paired *t*-test.

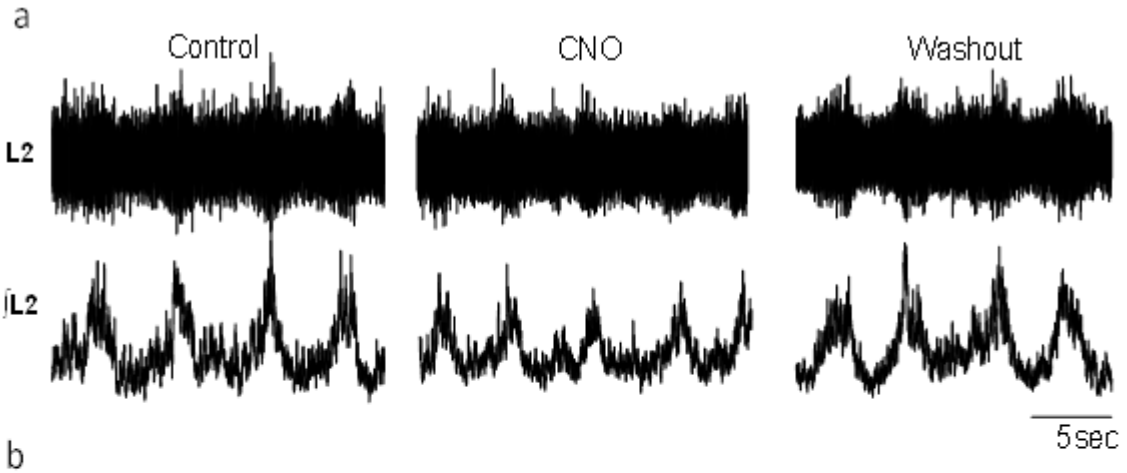
4.2.8. Chemogenetic inhibition of Pitx2^{+} INs during fictive locomotion decreases ventral root burst amplitude

Next, the effects of DREADD-based inhibition of Pitx2^{+} INs on population level MN output were assessed. This was achieved by performing ventral root recordings from spinal cords isolated from Pitx2-Cre;CHRM4 mice during pharmacologically-induced (NMDA, DA and 5-HT) locomotor activity. Pitx2^{+} INs were inhibited via perfusion of CNO. As seen in figure 4.25, inhibition of Pitx2^{+} INs during fictive locomotion decreased ventral root burst amplitude (control: 0.803±0.105; CNO: 0.715±0.096; washout: 0.779±0.115; n=14; $p<0.05$ one-way ANOVA with Tukey's post-test). However, inhibition of Pitx2^{+} INs had no significant effects on burst duration (control: 2252±618ms; CNO: 1893±557ms; washout: 1829±504ms; n=14), burst frequency (control: 0.229±0.033Hz; CNO: 0.229±0.034Hz; washout: 0.251±0.038Hz; n=14) or burst frequency variance (control: $5.560\times10^{-3}\pm2.283\times10^{-3}Hz^2$; CNO: $7.547\times10^{-3}\pm1.984\times10^{-3}$ Hz^2; washout: $8.744\times10^{-3}\pm3.812\times10^{-3}Hz^2$; n=14).

In CHRM4 only animals, which lack DREAD expression, CNO had no significant effects on locomotor-related ventral root burst frequency (control: 0.227±0.038Hz; CNO: 0.240±0.041Hz; washout: 0.297±0.056Hz; n=8), frequency variance (control: $7.320\pm2.390\times10^{-3}Hz^2$; CNO: $6.816\times10^{-3}\pm2.012\times10^{-3}Hz^2$; washout: $7.637\times10^{-3}\pm1.963\times10^{-3}Hz^2$; n=8), duration (control: 2074±555ms; CNO: 1639±366ms; washout: 1604±349ms; n=8) or amplitude (control: 0.630±0.118; CNO: 0.626±0.103; washout: 0.553±0.110; n=8) (figure 4.26). This confirms that CNO has no non-specific actions on spinal neurons and spinal locomotor network output.

These data demonstrate that Pitx2^{+} INs are active during locomotion and that their activity contributes to an intensification of the strength of locomotor-related motor

output. Meanwhile, Pitx2^{+} INs appear to have little influence on rhythm or pattern generating elements of spinal motor circuits.


a
Control
CNO
Washout
L2
∫L2
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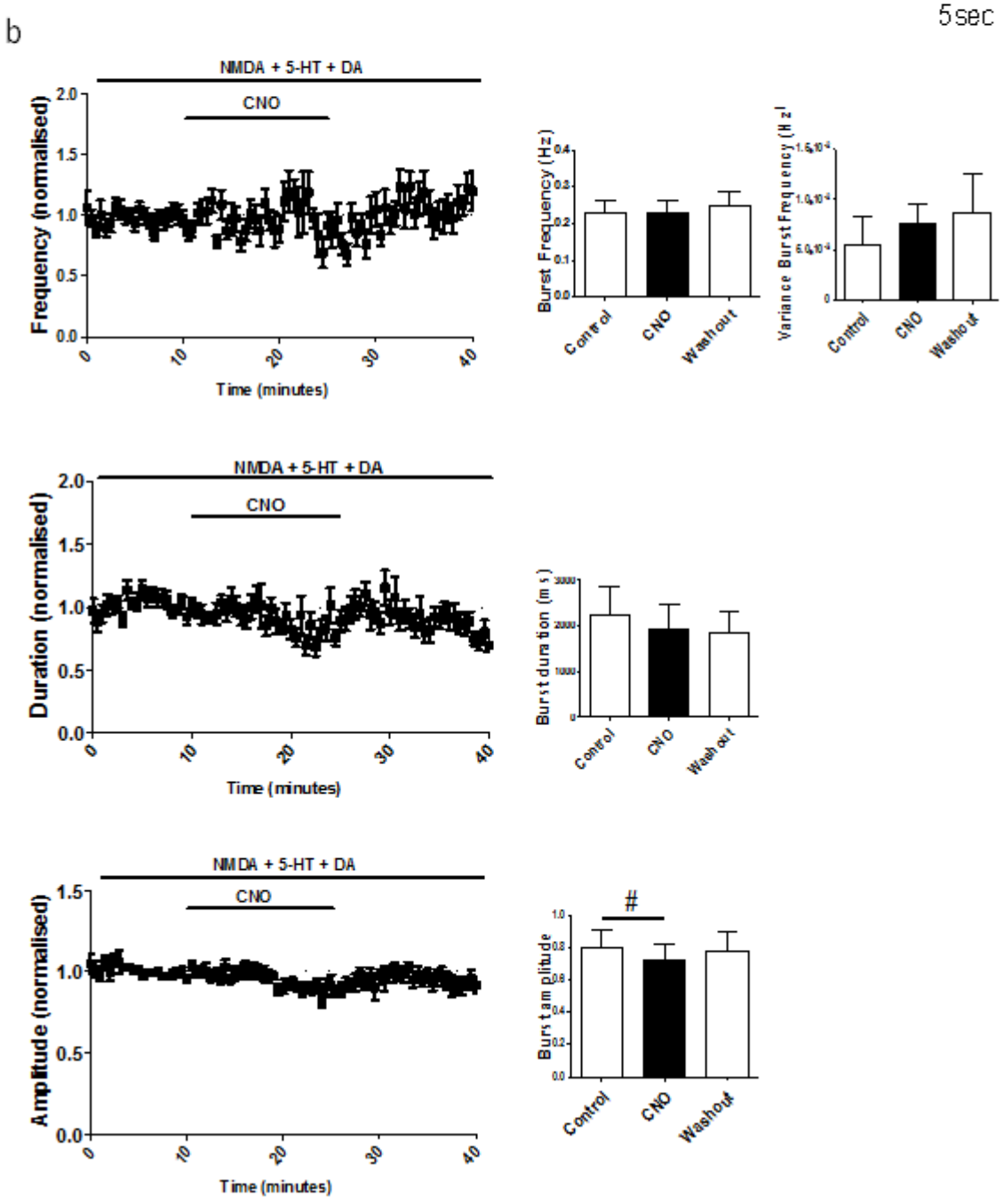

b
NMDA + 5-HT + DA
CNO
Frequency (normalised)
Time (minutes)
Burst Frequency (Hz)
Variance Burst Frequency (Hz²)
Control
CNO
Washout
NMDA + 5-HT + DA
CNO
Duration (normalised)
Time (minutes)
Burst duration (ms)
Control
CNO
Wash out
NMDA + 5-HT + DA
CNO
Amplitude (normalised)
Time (minutes)
#
Burst amplitude
Control
CNO
Washout

Figure 4.25 – Inhibition of Pitx2^{+} INs during fictive locomotion decreases burst amplitude **(a)** raw (top) and integrated/rectified (bottom) traces and **(b)** averaged time course plots (left) and histograms of pooled data (right) showing the effects of inhibition of Pitx2^{+} INs (CNO 1µM) on ventral root output (n= 14); #$p<0.05$ repeated measures ANOVA with Tukey's post-test

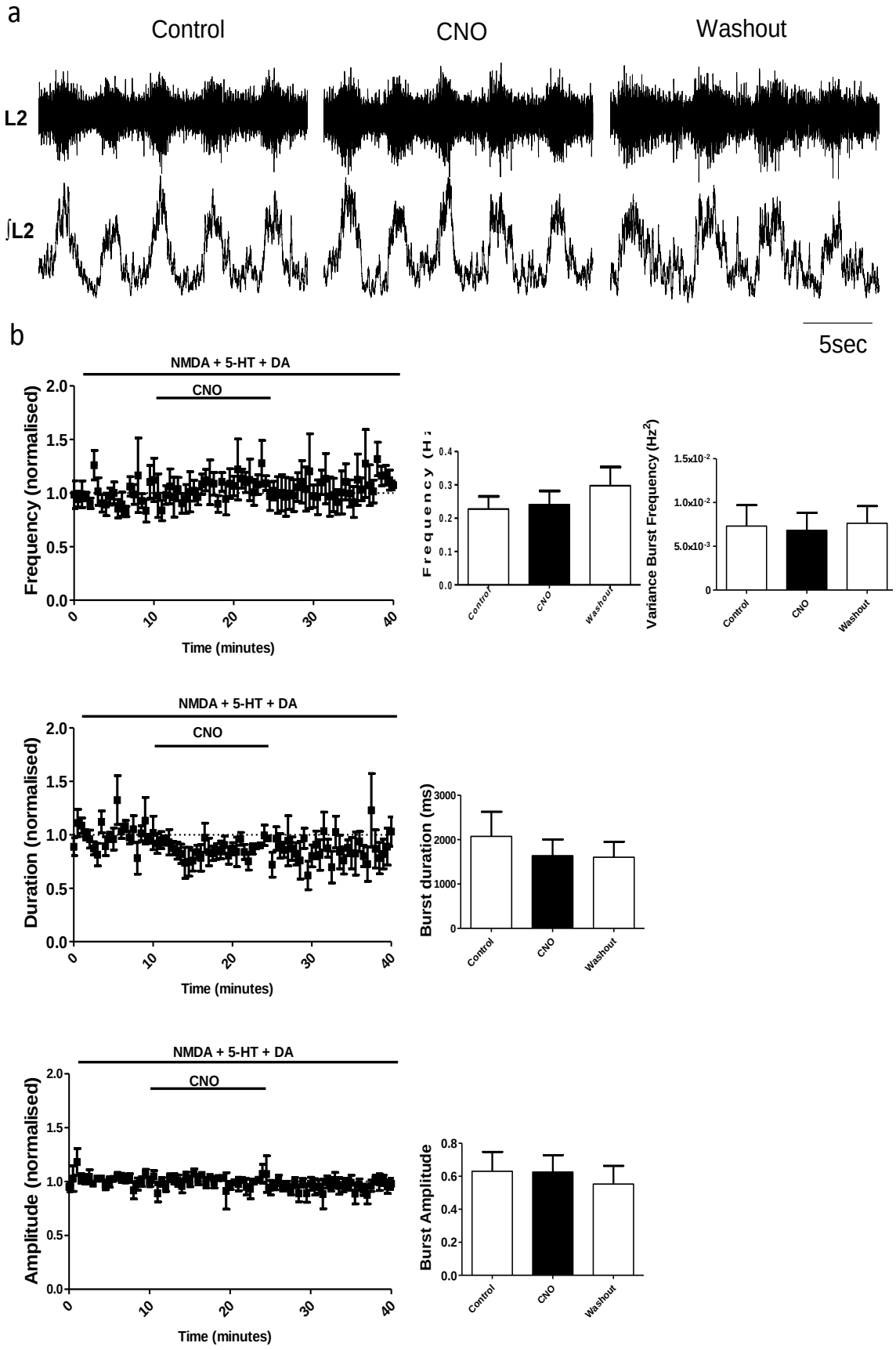

a
Control
CNO
Washout
L2
∫L2
5sec
b
NMDA + 5-HT + DA
CNO
Frequency (normalised)
Time (minutes)
Frequency (Hz)
Variance Burst Frequency (Hz²)
Control
CNO
Washout
NMDA + 5-HT + DA
CNO
Duration (normalised)
Time (minutes)
Burst duration (ms)
Control
CNO
Washout
NMDA + 5-HT + DA
CNO
Amplitude (normalised)
Time (minutes)
Burst Amplitude
Control
CNO
Washout

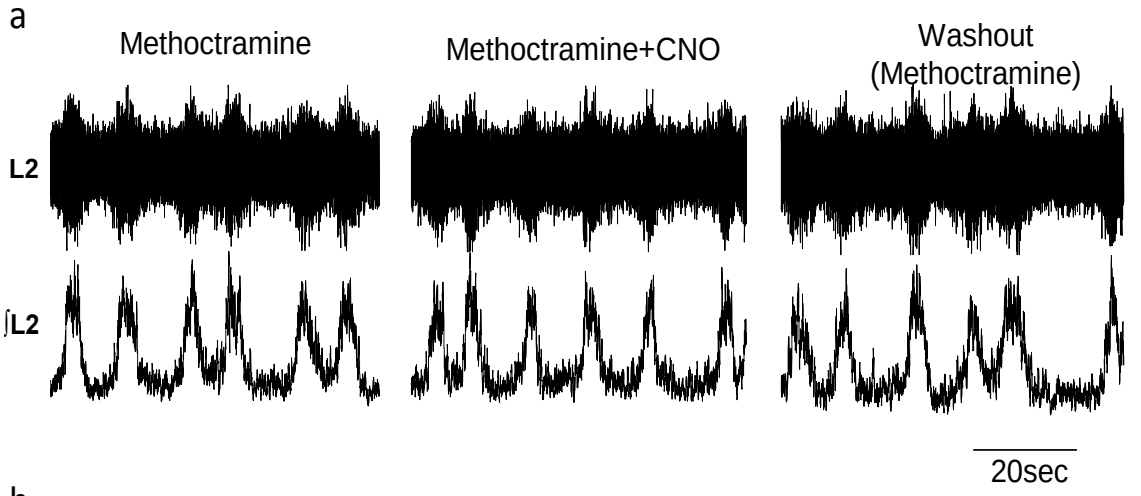
Figure 4.26 – CNO has no unspecific effects on ventral root output in control mice. **(a)** raw (top) and integrated/rectified (bottom) traces with **(b)** averaged time course plots (left) and mean pooled data (right) showing no effect of CNO (1μM) in CHRM4 mice drug-induced locomotor output (n=8). $p>0.05$ repeated measures ANOVA

4.2.9. The regulation of locomotor-related MN output by Pitx2$^+$ INs involves M2 muscarinic receptors

The role of M2 muscarinic receptors, found at C-bouton synapses, in the Pitx2$^+$ IN-mediated modulation of locomotor output was next investigated. CNO was applied in the presence of the M2 receptor antagonist methoctramine, to isolated spinal cord preparations from Pitx2Cre;CHRM4 mice during pharmacologically-induced, fictive locomotion. DREADD-mediated inhibition of Pitx2$^+$ INs in the presence of methoctramine (figure 4.27) had no significant effects on burst frequency (methoctramine: 0.281±0.057Hz; methoctramine and CNO: 0.242±0.053Hz; washout (methoctramine): 0.255±0.050Hz; n=8), frequency variance (methoctramine: $4.504\times10^{-3}\pm2.383\times10^{-3}Hz^2$; methoctramine and CNO: $7.653\times10^{-3}\pm3.666\times10^{-3}Hz^2$; washout (methoctramine): $4.873\times10^{-3}\pm1.117\times10^{-3}Hz^2$; n=8), burst duration (methoctramine: 2644±1023ms; methoctramine and CNO: 2880±1213ms; washout (methoctramine): 2682±1094ms; n=8) or burst amplitude (methoctramine: 0.935±0.134; methoctramine and CNO: 0.912±0.137; washout (methoctramine): 0.900±0.128; n=8).

The above results further confirm that M2 muscarinic receptors at the C-bouton synapse are involved in the modulation of MN function. Blockade of M2 receptors removed the Pitx2$^+$ IN-mediated modulation of MN output and as a result CNO-induced inhibition of Pitx2$^+$ INs no longer decreased ventral root burst amplitude. This is in line with the results with methoctramine from previous experiments in Pitx2-Cre;CHRM3

mice (section 4.2.2) highlighting the involvement of M2 muscarinic receptors as the key target underlying cholinergic modulation from this subset of spinal INs.


a
Methoctramine
Methoctramine+CNO
Washout
(Methoctramine)
L2
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20sec

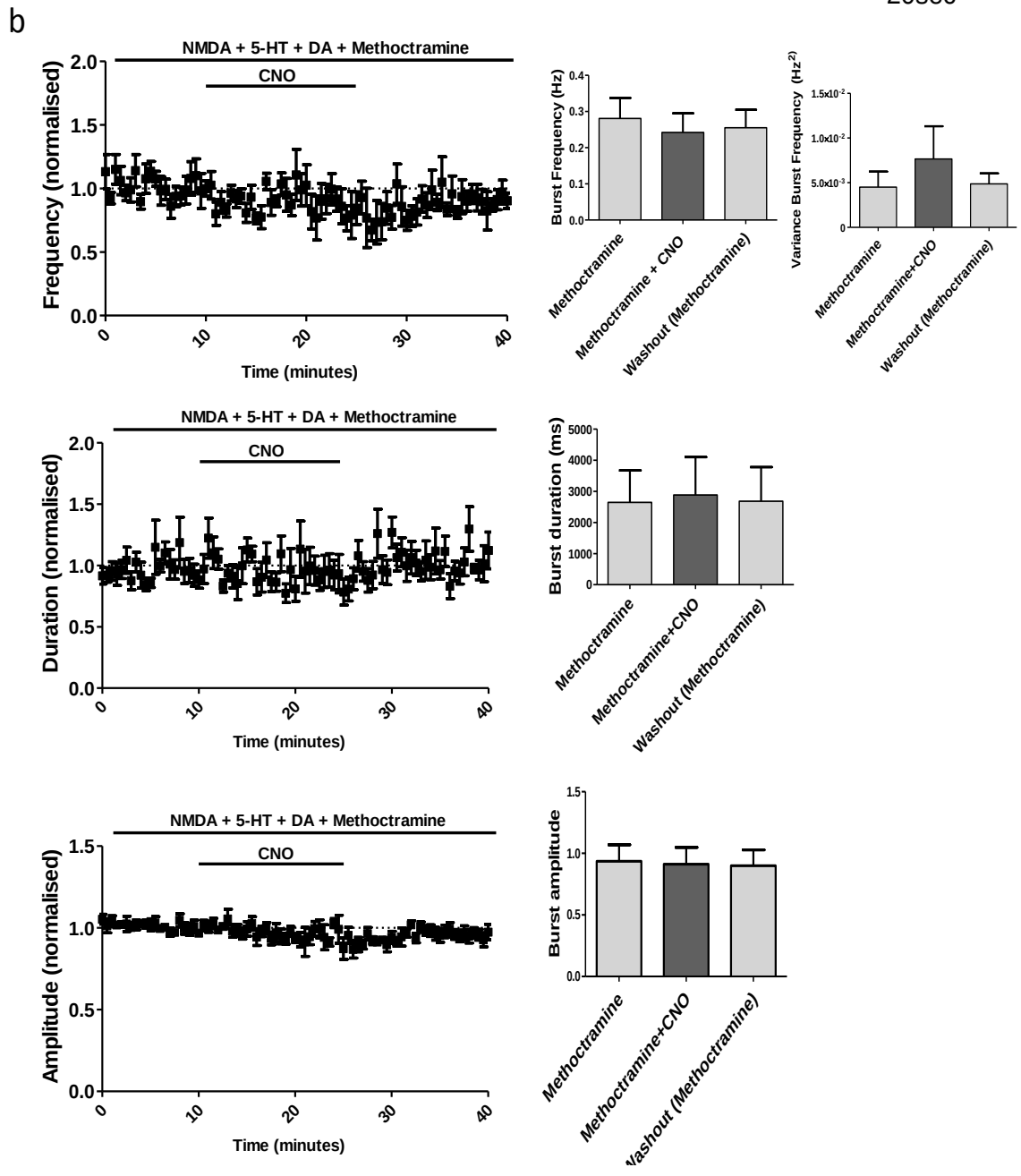

b
NMDA + 5-HT + DA + Methoctramine
CNO
Frequency (normalised)
Time (minutes)
Burst Frequency (Hz)
Variance Burst Frequency (Hz²)
Methoctramine
Methoctramine + CNO
Methoctramine+CNO
Washout (Methoctramine)
Duration (normalised)
Burst duration (ms)
Amplitude (normalised)
Burst amplitude

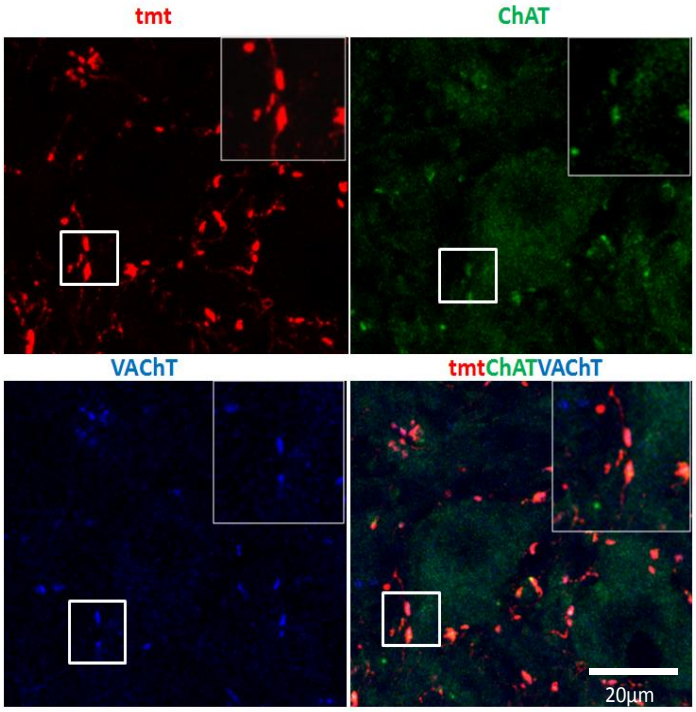
Figure 4.27 - DREADD-mediated inhibition of Pitx2$^+$ INs during fictive locomotion is dependent on M2 receptors. **(a)** raw (top) and integrated/rectified (bottom) traces with **(b)** averaged time course plots (left) and mean pooled data (right) showing the effect of prior blockade of M2 receptors with methoctramine (10μM) on the inhibitory actions of CNO (1μM) in Pitx2-Cre::CHRM4 mice drug-induced locomotor output (n=8). $p>0.05$ repeated measures ANOVA

4.3. Genetic ablation of Pitx2$^+$ INs and C-boutons reveals M2 muscarinic receptor-mediated modulation of the intensity of locomotor-related output

Results from the previous sections indicate that Pitx2$^+$ INs modulate MN output via M2 receptors found at C-bouton synapses, with activity of Pitx2$^+$ INs causing an increase in MN output. Genetic ablation of interneuronal populations in the spinal cord has proven useful to fully understand the role of specific types of INs in locomotor circuitry (Goulding, 2009; Arber, 2012). The effects of ablating cholinergic Pitx2$^+$ INs on locomotor network output was therefore next assessed.

Pitx2::Cre mice were crossed with novel VAChT-*loxP-STOP-loxP-DTA* animals in order to achieve conditional ablation of cholinergic Pitx2$^+$ INs via diphtheria toxin A, whose Cre-inducible expression has been widely used for *in vivo* genetic deletion of spinal INs (Crone *et al.*, 2008). As illustrated in figure 4.28, 25 day old Pitx2-Cre;TdTomato;DTA mice exhibit a clear reduction of C-boutons to MNs. This was calculated as a 92.3% loss of cholinergic Pitx2$^+$ INs, indicating a high degree of efficiency in the conditional ablation of these INs (figure and quantification courtesy of Maria Mina and Laskaro Zagoraiou, Academy of Athens).

Pitx2-Cre;TdTomato



Pitx2-Cre;TdTomato;DTA

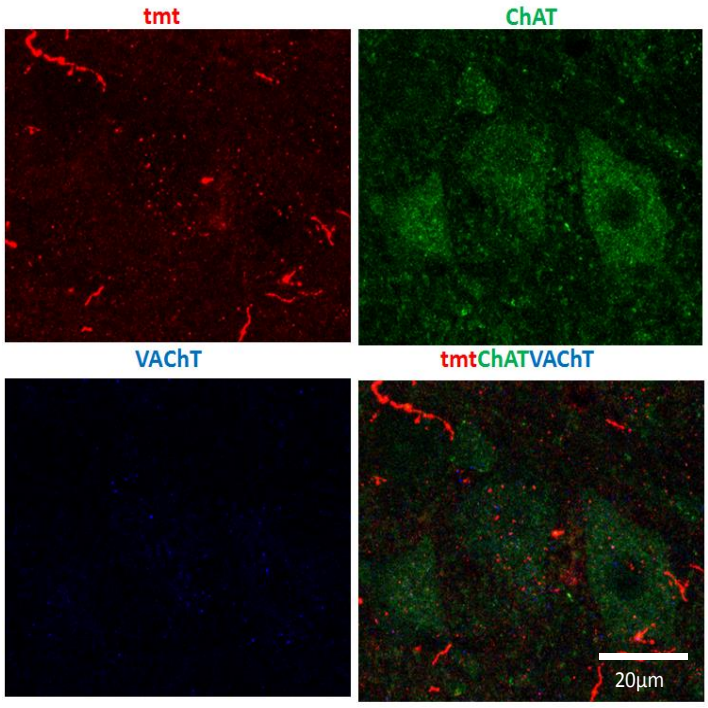
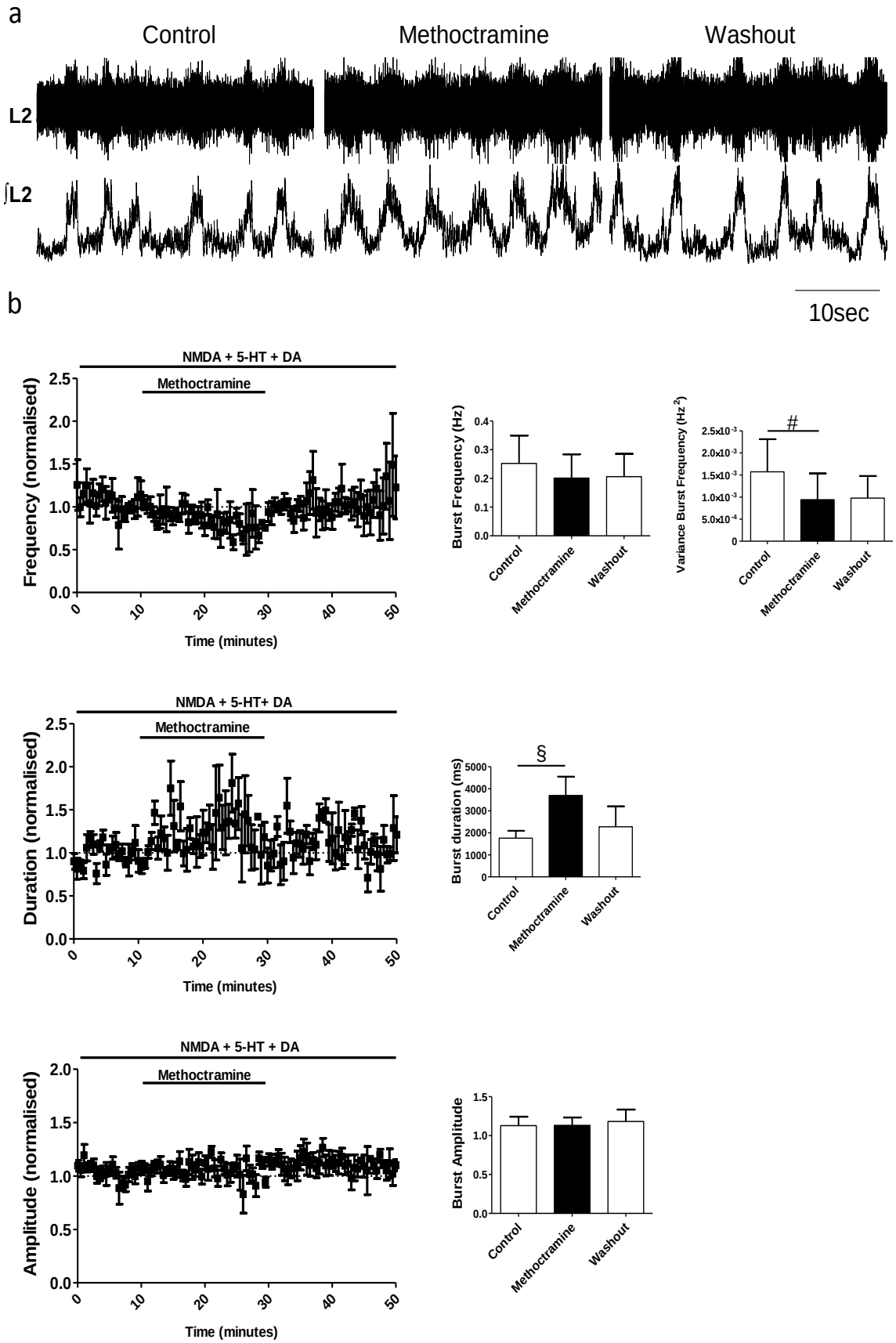


Figure 4.28 – Genetic ablation of cholinergic Pitx2^{+} INs eliminates C-boutons around MN soma. Spinal cord from a 25 days old Pitx2-CreTdTomato;DTA (bottom) and age-matched control Pitx2-Cre;TdTomato mice (top), depicting the lack of cholinergic C-boutons to MNs. Tmt – TdTomato fluorescent reported; ChAT – choline acetyltransferase; VAChT – vesicular acetylcholine transporter; C-boutons are highlighted in white boxes (top). Figure is a courtesy of Maria Mina and Laskaro Zagoraiou, Academy of Athens.

The effects of the genetic ablation of cholinergic Pitx2^{+} INs, and their C-boutons, was assessed during pharmacologically-induced locomotion in spinal cord preparations from Pitx2-Cre;DTA mice. The M2 antagonist methoctramine was perfused to investigate if the previously described decrease in burst amplitude (section 4.1.1) was removed when Pitx2^{+} INs and their C boutons were absent. As illustrated in figure 4.29, bath application of methoctramine in Pitx2-Cre;DTA mice significantly decreased burst frequency variance (control: $1.572\times10^{-3}\pm7.370\times10^{-4}Hz^2$; methoctramine: $9.403\times10^{-4}\pm5.946\times10^{-4}Hz^2$; washout: $9.762\times10^{-4}\pm4.990\times10^{-4}Hz^2$; n=4; $p<0.05$ repeated measures ANOVA with Tukey's post-test) and increased burst duration (control: 1757±332ms; methoctramine: 3696±850ms; washout: 2274±924ms; n=4; $p<0.05$ Friedman's test with Dunn's post-test). However, the M2 receptor antagonist had no significant effect on burst frequency (control: 0.252±0.097Hz; methoctramine: 0.201±0.083Hz; washout: 0.206±0.079Hz; n=4) or burst amplitude (control: 1.128±0.115; methoctramine: 1.132±0.102; washout: 1.182±0.152; n=4).

The data from these experiments suggest that Pitx2^{+} INs and their C-bouton contacts with MNs are solely responsible for the M2 muscarinic receptor-mediated modulation of the intensity of locomotor-related output. In addition, these data suggest that the additional effects of M2 receptor activation on locomotor network function do not involve Pitx2^{+} INs or C-bouton synapses.


a
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NMDA + 5-HT + DA
Methoctramine
Frequency (normalised)
Time (minutes)
Burst Frequency (Hz)
Control
Methoctramine
Washout
Variance Burst Frequency (Hz²)
#
NMDA + 5-HT+ DA
Duration (normalised)
Burst duration (ms)
§
NMDA + 5-HT + DA
Amplitude (normalised)
Burst Amplitude

Figure 4.29 – Genetic ablation of Pitx2^{+} INs in Pitx2-Cre;DTA mice removes inhibitory effect on burst amplitude elicited by methoctramine. **(a)** raw (top) and integrated/rectified (bottom) traces with **(b)** averaged time course plots (left) and mean pooled data (right) illustrating the effects of methoctramine (10μM) on Pitx2-Cre;DTA ventral root output (n=4); $p<0.05$ repeated measures ANOVA with Tukey's post-test. § $p<0.05$ Friedman's test with Dunn's post-test

5. DISCUSSION

5.1. Muscarinic modulation of spinal locomotor networks and MN function in the neonatal mouse spinal cord

ACh is released during locomotion (Huang *et al.*, 2000; Dai *et al.*, 2009; Dai and Jordan, 2010) acting as a neuromodulator of CPG networks in mammals (Cowley and Schmidt, 1994; Miles *et al.*, 2007; Jordan *et al.*, 2014). High concentrations of ACh or muscarine in the neonatal rat spinal cord can increase network excitability which can result in the generation of bursts of motor activity, however these can be erratic with no stable right-left alternation or extensor-flexor phase relationship for long-lasting periods of time (Cowley and Schmidt, 1994; Kiehn *et al.*, 1996; Jordan *et al.*, 2014). In the mouse spinal cord exogenous application of muscarine evokes irregular ventral root discharges with no locomotor-like pattern of activity (Jiang *et al.*, 1999). The increase in the levels of ACh in the mammalian spinal cord during locomotion does not appear to be involved in the initiation and establishment of appropriate rhythmic CPG activity but instead in the fine-tuning of spinal locomotor networks through the activation of mAChRs (Cowley and Schmidt, 1994; Miles *et al.*, 2007; Dai *et al.*, 2009; Dai and Jordan, 2010; Jordan *et al.*, 2014). This characteristic confers ACh a powerful modulatory role in spinal locomotor circuitry that required further understanding.

In order to study the effects of muscarinic receptors on locomotor network output, experiments were performed in neonatal mouse spinal cords in the presence of NMDA, 5-HT and DA that induced locomotor-related bursts of rhythmic activity stable for long periods of time and that exhibit adequate right-left and extensor-flexor alternation (Jiang *et al.*, 1999). Considering that M2 and M3 muscarinic receptors are widely expressed in the spinal cord (Oguz Kayaalp and Neff, 1980; Welton *et al.*, 1999;

Wilson *et al.,* 2004) and appear to be solely responsible for cholinergic modulation of locomotor output in isolated rat spinal cord preparations in which ACh levels were enhanced by blocking ACh esterase activity (Jordan *et al.,* 2014), selective antagonists of these two muscarinic receptors were perfused to study the role of each receptor subtype in the modulation of rhythmically active CPG networks and MN output in mice. This is the first study that used both muscarinic receptor antagonists in drug-induced neonatal mouse spinal cord preparations to study endogenous cholinergic modulation. In these preparations the levels of ACh were not manipulated nor was endogenous ACh used to elicit rhythmic bursts, thus providing a more physiological readout and allowing to isolate the effects of each antagonist on the CPG network and MN output during *in vitro* locomotor patterns that resemble walking (Jiang *et al.,* 1999). M2 muscarinic receptor antagonism decreased the variability of the locomotor bursts while also reducing the amplitude of the ventral root signal. This indicates that activation of M2 receptors is responsible for modulation of CPG INs involved in the generation of the locomotor rhythm as well as modulation of properties of MNs that define the strength of the motor output (section 5.1.1). Pharmacological blockade of M3 muscarinic receptors destabilized the drug-evoked bursting without a significant effect on burst amplitude, indicating that these receptors are important for adequate rhythm assembly during fictive locomotion without significantly affecting MN output (section 5.1.2).

To explore changes in cellular properties following muscarinic receptor activation, single cell recordings were performed from MNs within spinal cord slices. MNs are the last target in the locomotor network before initiation of muscle contraction and therefore insights regarding the impact of muscarinic receptor activation on these

cells will provide valuable information about cholinergic modulation of motor output. Exogenous application of muscarine was found to effect subthreshold properties, firing output of MNs and synaptic drive to MNs. These effects were found to exclusively dependent on M2 and M3 muscarinic receptors. M2 receptor activation elicited an outward current, decreased the mAHP amplitude possibly contributing to increased MN output and decreased the frequency of synaptic inputs to MNs (section 5.1.3). M3 receptor activation caused an inward current, influenced MN maximum firing output and increased synaptic inputs to MNs (section 5.1.4). Interestingly, M2 and M3 muscarinic receptors seem to have opposite roles on the modulation of CPG networks and MN function in the mouse lumbar spinal cord, indicating that a balance between M2 and M3 receptor actions is present (section 5.1.5).

The interpretation of the results obtained was based on the pharmacological effectiveness of the general agonist, muscarine, and the selective M2 and M3 antagonists, methoctramine and 4-DAMP, respectively. The development of selective agonists for muscarinic receptors has been slowed down by the highly conserved orthosteric binding site (i.e. active binding site) among the different subtypes of receptors due to the shared homology in the transmembrane domains with some of the antagonists also displaying concentration-dependent lack of specificity in allosteric regulation. This makes the use of muscarinic receptor agonists limited to non-selective agonists, with selective antagonists being used to discriminate receptor specificity (Korczynska *et al.*, 2018). Methoctramine has high affinity for the M2-receptor with approximately 16 times less binding affinity to the M3 receptor (Korczynska *et al.*, 2018). Some studies have suggested that methoctramine might also have high affinity for the M4 muscarinic receptor subtype (M. Waelbroeck *et al.*, 1990), with radioligand

binding experiments pointing to the presence of this receptor in the dorsal horn of the spinal cord (Höglund and Baghdoyan, 1997). Neurochemistry and electrophysiological experiments in double M2/M4-knockout studies performed in the mouse spinal cord have implicated the M4 receptor subtype in nociception but not in the control of locomotor networks (H. M. Zhang, Zhou, *et al.*, 2007; Zhang *et al.*, 2009; Chen *et al.*, 2014). However due to the poor selective affinity of M4 receptor ligands, the exact role of this receptor in spinal nociception is still unclear. Hence, the actions of methoctramine in this work will be discussed as a pharmacological block of only M2 muscarinic receptors. 4-DAMP has a preference for the M3 muscarinic receptor subtype but can also exhibit some affinity for the M1 receptor subtype (Eglen *et al.*, 1994; Ehlert, 1996). The M1 subtype does not seem to be present in the spinal cord (Höglund and Baghdoyan, 1997) thus the interpretation of the results with 4-DAMP will be focused exclusively on the M3 muscarinic receptor.

5.1.1. Modulation of spinal locomotor output by M2 muscarinic receptors

The M2 muscarinic receptor antagonist, methoctramine, decreased the variance of burst frequency and increased burst duration indicating that these receptors are involved in setting up the regularity of the drug-induced locomotor rhythm. Experiments with M2 blockers in ACh-induced bursts of activity in rat spinal cord preparations showed that methoctramine increased the frequency of locomotor events (Jordan *et al.*, 2014). Also in the neonatal rat spinal cord, experiments stimulating sacral dorsal roots while simultaneously increasing the levels of ACh in the sacral region with ACh-esterase inhibitors, decreased the frequency of bursts of activity recorded from

lumbar ventral roots, an effect that was blocked by methoctramine (Anglister *et al.*, 2017). The current observations from ventral root recordings in the presence of NMDA, DA and 5-HT with methoctramine in mice do not show a statistically significant difference in burst frequency between control and drug but report an increase in burst duration by methoctramine which could be indicative of a slowing of the rhythm. The differences between these results and the observations from other authors (Jordan *et al.*, 2014; Anglister *et al.*, 2017) could indicate that (1) there are functional differences on M2 muscarinic receptor modulation of the frequency of spinal motor output between mouse and rat or (2) these modulations are different between NMDA, DA and 5-HT induced rhythmogenesis and locomotor activity generated in the presence of ACh-esterase inhibitors, which will greatly increase the levels of ACh present. As mentioned before, there are indeed differences regarding cholinergic-induced locomotion between mouse and rat, with perfusion of muscarine eliciting regular bursts of activity in the rat spinal cord but not in mice (Kiehn *et al.*, 1996; Jiang *et al.*, 1999). Another important factor is that in the present work rhythmic bursting evoked with the locomotor cocktail is more regular and exhibits phase alternation between right-left and extensor-flexor output, contrary to the experiments performed in the rat spinal cord (Jordan *et al.*, 2014). In addition, the concentration of released ACh during alternating fictive locomotion might be more physiological than when in the presence of high concentrations of ACh elicited by ACh-esterase inhibitors or general cholinergic agonists, as illustrated by experiments in the mudpuppy spinal cord in which carbacol (ACh analog) and physostigmine disrupted the fictive walking rhythm induced by NMDA (Fok and Stein, 2002). These differences could underlie changes in M2 receptor activation and/or network excitability that might explain the variations in the results on

burst frequency with methoctramine when comparing the current results with previous work (Jordan *et al.*, 2014; Anglister *et al.*, 2017). Despite these incongruities in the experimental setup, recordings performed in the rat lumbar spinal cord also indicated that M2 receptor antagonism reduced burst amplitude (Jordan *et al.*, 2014; Anglister *et al.*, 2017) which mirrors the decrease in ventral root amplitude elicited by methoctramine that is reported in this work. This suggests that there is a level of conservation regarding M2 muscarinic receptor effects on motor output amplitude in mouse and rat, even with the different approaches used, indicating that activation of these receptors increases the strength of locomotor output.

Groups of cholinergic INs in the spinal cord could be responsible for the reported muscarinic effects on the locomotor network. However, apart from Pitx2^{+} INs, there remain a lack of genetic markers for subtypes of spinal cholinergic INs. Researchers proposed that cholinergic neurons from sacral segments that project to the lumbar region could directly modulate CPG networks and MN output (Etlin *et al.*, 2014; Finkel *et al.*, 2014; Anglister *et al.*, 2017). These sacral projecting cholinergic neurons could include a variety of different populations of premotor ACh-releasing INs that are known to project intersegmentally (Stepien *et al.*, 2010). Most of the cholinergic INs that could be involved in modulation of CPG-mediated rhythmogenesis are located in the ventromedial area of the spinal cord and they comprise Pitx2^{+} INs, partition cells and contralaterally projecting INs that form synapses with MNs and other spinal INs (Sherriff and Henderson, 1994; Huang *et al.*, 2000; Zagoraiou *et al.*, 2009; Bertrand and Cazalets, 2011). Pitx2^{+} INs are suggested to directly modulate MN output through M2 muscarinic receptors (Miles *et al.*, 2007; Zagoraiou *et al.*, 2009) thus, they are likely to be responsible for the decrease in burst amplitude and MN firing caused by

methoctramine. The potential roles of these INs in drug-induced locomotion will be addressed in later sections discussing the results from DREADD experiments in which Pitx2^{+} INs were selectively activated and inhibited (section 5.2).

Phase alternation between antagonistic muscles and right-left sides is crucial for adequate spinal locomotor activity (Brown, 1911). This can be replicated during *in vitro* drug-induced locomotor-bursts of activity in the neonatal mouse spinal cord allowing to study effects of modulators that may affect these phase relationships (Jiang *et al.*, 1999). This study did not systematically measure any eventual variations in extensor-flexor or right-left alternation, however a clear breakdown of these relationships upon blockade of muscarinic receptors was not observed. The data obtained during fictive locomotion reports changes in ventral root burst duration, frequency and variance in the presence of the antagonists used, which could reflect a modulation of different types of CPG INs in the mouse spinal cord. In V1 knockout mice, the duration of the bursts during stepping behaviour was decreased (Gosgnach *et al.*, 2006). V1 INs do not express cholinergic markers however they receive some cholinergic innervation from primary afferents (Alvarez *et al.*, 2005), which could suggest that M2 muscarinic receptors that are expressed in dorsal INs and control presynaptic release from these afferents (Stewart and Maxwell, 2003; Wang *et al.*, 2006; H. M. Zhang, Chen, *et al.*, 2007; H. M. Zhang, Zhou, *et al.*, 2007; H. Zhang *et al.*, 2007) could be acting directly or having an indirect network effect on this population of CPG INs that could be responsible for observed increases in burst duration. V2a INs that control right-left alternation in mice (Crone *et al.*, 2008) have sparse cholinergic innervation (Zagoraiou *et al.*, 2009). Furthermore, V2a genetic ablation did not change cycle period, amplitude or duration of bursts (Crone *et al.*, 2008). Thus, it is unlikely that cholinergic modulation of the locomotor

CPG involves this IN subpopulation. The dI6 INs are located around the ventromedial area of the spinal cord, are active during locomotion and seem to be important for the formation of rhythmic patterns (Lanuza *et al.*, 2004; Dyck *et al.*, 2012). The clustering of cholinergic INs in ventromedial regions (Bertrand and Cazalets, 2011) might facilitate local cholinergic modulation of INs near laminas X and VII involved in stabilizing the locomotor rhythm, which could include populations such as dI6 INs. Due to their sparse distribution in the spinal cord (Wilson *et al.*, 2004), it is plausible that the effects of M2 muscarinic receptors on the drug-induced rhythm are a reflection of a modulation on several types of INs that form the locomotor CPG rather than an action on one particular type of IN.

5.1.2. Modulation of spinal locomotor output by M3 muscarinic receptors

There has been less previous work defining the role of M3 muscarinic receptors in spinal CPG circuits. In the present study blockade of M3 receptors disrupted the drug-induced locomotor rhythm as evidenced by an increase in the variance of burst frequency and a decrease in burst duration. There were no significant changes in the amplitude of locomotor-related bursts nor MN firing suggesting that M3 muscarinic receptors do not have a role in the modulation of MN output during fictive locomotion. During ACh-induced bursts of activity in the neonatal rat spinal cord, application of the M3 receptor antagonist 4-DAMP decreased burst frequency and could stop ventral root activity at concentrations in the nanomolar range. Based on this, the authors suggested that M3 muscarinic receptors were responsible for the endogenous action of ACh in the generation of motor bursting (Jordan *et al.*, 2014). There is some evidence that these

receptors are expressed in laminas VII, VII and X (Wilson *et al.*, 2004) suggesting that M3 muscarinic receptors could influence CPG neurons in the ventral horn. Activation of this receptor subtype could therefore affect firing properties of CPG INs involved in stabilization of the rhythm such as dI6 (Lanuza *et al.*, 2004; Dyck *et al.*, 2012) and V1 INs (Alvarez *et al.*, 2005; Gosgnach *et al.*, 2006), as previously discussed for the M2 muscarinic receptor. With very limited studies focused on M3 muscarinic receptors in the spinal cord, this is the first time a functional description for this receptor subtype has been highlighted during *in vitro* alternating hindlimb locomotion. The few studies on M3 receptor distribution in the spinal cord (Wilson *et al.*, 2004) and mechanism of action (Bertrand and Cazalets, 2011; Jordan *et al.*, 2014) limit interpretation of the findings of the current work. M3 muscarinic receptors can inhibit the M-current which results in increased neuronal excitability (Brown and Passmore, 2009; Bertrand and Cazalets, 2011), however no study has addressed the role of this current in spinal locomotion. Blocking M3 muscarinic receptors with 4-DAMP could have removed the cholinergic downregulation of the M-current which would decrease the activity of some CPG INs that are key in maintaining burst regularity. A recent study has shown that in the rat parafacial respiratory group, which is an important expiratory oscillator, activation of M3 muscarinic receptors increased the excitation of rhythmic neurons which in turn recruited adequate expiratory activity (Boutin *et al.*, 2017). Previous work done in the neonatal rat suggested that in a medullary region of the brainstem that contains neurons responsible for the generation of the respiratory rhythm - the preBötzinger complex - M3 muscarinic receptors are helpful to ensure adequate frequency of respiratory patterns by modulating the activity of neurons important in rhythm generation (Shao and Feldman, 2000, 2005). Considering that M3 muscarinic

receptors seem to be important in modulating rhythmicity in the respiratory system (Shao and Feldman, 2000, 2005; Boutin *et al.*, 2017) and the phylogenetical and functional similarities between brainstem and spinal CPGs (Smith *et al.*, 2013; Berg, 2017) it may follow that M3 receptor modulation in the locomotor circuitry at the lumbar region is also important for ensuring adequate rhythmic patterns of activity. Results from this thesis indicate that M3 muscarinic receptors are important in maintaining rhythm stability during fictive locomotion.

5.1.3. Modulation of MN function by M2 muscarinic receptors

To explore changes in the cellular properties of MNs induced by muscarinic receptor activation, whole-cell patch clamp recordings were performed and MN function was studied. When investigating whether muscarinic receptor activation had any subthreshold effects on MNs that might modulate their resting membrane potential, two different types of changes in current were observed upon application of muscarine, either an inward or an outward current. Activation of M2 muscarinic receptors was responsible for the outward current in MNs. Similar results have been reported in sympathetic preganglionic spinal INs in which authors observed that M2 receptor activation decreased input resistance and elicited a hyperpolarization whose magnitude was reduced by lowering the extracellular levels of K^+ (Gibson and Logan, 1995). Interestingly all MNs tested seemed to have a M2 muscarinic receptor-dependent outward current in the presence of the M3 receptor antagonist, however when the general agonist was perfused alone this feature was only detected in a subset of smaller MNs. This could reflect diverse expression of M2 receptors in different MNs or a balance between M2 and M3 receptors (see section 5.1.5). Immunolabelling studies

have suggested a preferential expression of M2 muscarinic receptors by large MNs with some smaller MNs displaying weak labelling (Welton *et al.*, 1999), therefore differential expression of M2 receptors on MN soma is unlikely to explain the predominance of M2 receptor-mediated responses in smaller MNs. M2 muscarinic receptors are also expressed by cholinergic INs scattered around the dorsal and ventromedial areas of the spinal cord (Stewart and Maxwell, 2003; Wilson *et al.*, 2004) which raises the possibility of a network effect being responsible for the M2 muscarinic receptor-mediated changes in current. However, given that M2 receptor-mediated outward currents were present when evoked activity was blocked with TTX, just like in preganglionic INs (Gibson and Logan, 1995), it seems that these currents reflect activation of M2 receptors on MNs. The reversal potential for K^+ in the solutions used is -98mV whereas the reversal for Cl^- is -62mV. The current-voltage relationship of the outward current measured in MNs in response to muscarine alone or muscarine co-applied with the M3 receptor antagonist in the presence or absence of TTX showed a reversal potential between -90 and -100mV, near to the reversal for K^+ in the solutions used. Thus, M2 receptor activation appears to induce a hyperpolarizing, possibly outward current in MNs by opening leak K^+ channels due to the linearity of the I-V trace. These channels help to regulate resting membrane potential by maintaining the voltage below the threshold for action potential initiation and their modulation can induce dynamic changes in cell excitability (Bockenhauer *et al.*, 2001). Leak K^+ channels have been shown to be regulated by a range of intrinsic modulators of spinal neurons, for example, adenosine-induced hyperpolarization in ventral horn INs occurs through A1 adenosine receptor activation that leads to the opening of leak K^+ channels (Witts *et al.*, 2015). In electrophysiological recordings from the salamander spinal cord,

muscarine hyperpolarized MNs and decreased input resistance with a slope that had a reversal value close to the equilibrium potential for K^+ (Chevallier *et al.*, 2006). This hyperpolarization was show to be mediated by I_{KIR} (Chevallier *et al.*, 2006) which could suggest that perhaps the presently described M2 muscarinic receptor effects on the holding current from mouse MNs could also result from a modulation of I_{KIR}. Future experiments with Ba^{2+} (I_{KIR} blocker) in the presence of 4-DAMP and muscarine would help to clarify this hypothesis.

Surprisingly, some MNs in which outward currents were observed when muscarine was perfused with 4-DAMP (in the presence or absence of TTX) had an increase in input resistance. The capacitance of these neurons was not statistically different than the MNs that displayed a decrease in input resistance. Despite overall M2 muscarinic receptor activation inducing an outward current, the opposing changes in input resistance in these 2 groups of MNs could underlie particular M2 receptor modulations that could arise from distinctive cholinergic inputs (see section 5.2.1) or coupling to different pathways and downstream channels in different MNs that might not directly interfere with M2 receptor-dependent changes in transient current.

In recordings from neonatal mouse MNs, muscarine increased MN output and decreased mAHP amplitude with both being blocked by methoctramine. These data are in line with results from previous work (Miles *et al.*, 2007). This particular modulation was presumed to be mediated by $Pitx2^+$ INs that form large C-boutons on MNs and activate postsynaptic M2 muscarinic receptors (Miles *et al.*, 2007; Zagoraiou *et al.*, 2009; Witts *et al.*, 2014). Immunolabelling studies indicated the presence of M2 receptors around the cell soma (Welton *et al.*, 1999) of MNs with intense postsynaptic clustering at C-bouton synapses (Wilson *et al.*, 2004). At the postsynaptic site in these

synapses there is evidence of other proteins that could be a target for M2 muscarinic receptor regulation such as small conductance Ca^{2+}-activated K^+ channel (SK) and Kv2.1 channels (Witts *et al.*, 2014). Modulation of SK channels by muscarine has been shown to account for decreased mAHP in spinal MNs in the salamander (Chevallier *et al.*, 2006) which could be part of the cholinergic mechanism involved in the increase of MN output in mice (Miles *et al.*, 2007). The particular mechanisms of MN modulation by M2 receptors and other proteins that are present at C-bouton synapses will be discussed in more detail in a later section which addresses the experiments performed using DREADD-mediated activation and inactivation of $Pitx2^+$ INs in mice (section 5.2.1). Since M2 receptors are also present on areas of the MN soma that are not juxtaposed of C-boutons (Welton *et al.*, 1999; Wilson *et al.*, 2004), other signalling pathways that do not comprise $Pitx2^+$ INs might be involved in the presently described cholinergic modulation of MN function. In the salamander spinal cord, authors found that although muscarine increased MN output, it also decreased the I_h current which usually leads to a decrease in spiking probability (Chevallier *et al.*, 2006). Blockade of I_h can induce a small hyperpolarization (Chevallier *et al.*, 2006, 2008) which is known to affect MN resting membrane potential (Kjaerulff and Kiehn, 2001). Activation of the M2 muscarinic receptor subtype is involved in the inhibition of I_h in cholinergic striatum neurons (Zhao *et al.*, 2016). Whether the M2 muscarinic receptor subtype also regulates I_h in mammalian MNs, thus affecting muscarine-induced increases in maximum firing and contributing to a hyperpolarization, could be investigated under the presence of low concentrations of Cs^+ that are known to block the I_h current (Chevallier *et al.*, 2006).

The increase in synaptic inputs, that is attributed to M3 receptor activation, was observed in the first minutes of perfusion and it was followed by a M2 receptor-mediated decrease in PSCs. Experiments performed by Bertrand & Cazalets (2011) have shown that electrical stimulation of the ventral commissure in spinal cord slices triggers the activation of neurons around the central canal area, where the majority of cholinergic INs seem to be located, which elicits synaptic potentials that can be recorded from MNs. In the presence of another M2 muscarinic receptor antagonist - AF-DX116 – the authors found a decrease in the amplitude and duration of these postsynaptic potentials, suggesting that M2 receptor activation increases network excitation of MNs. In the work from this thesis, recordings from MNs from isolated spinal cord slices showed that M2 muscarinic receptor activation decreased neuronal drive to MNs. Although the data clearly shows a modulation of synaptic activity, the PSCs could include excitatory and/or inhibitory inputs since the reversal for Cl^- in our solutions (-62mV) is close to the holding potential during voltage clamp recordings (-60mV). In the mid-lumbar spinal cord of rats around 37-45% of dorsal and lamina X cholinergic INs were found to express the M2 muscarinic receptor with the majority of these INs also expressing GABAergic and glycinergic markers (Stewart and Maxwell, 2003). In recordings from dorsal horn INs, activation of presynaptic M2 muscarinic receptors was suggested to increased GABA release through a phosphoinositide 3-kinase mediated pathway (Wang *et al.*, 2006; Zhang *et al.*, 2009) and inhibit glycine release to other spinal INs (H. M. Zhang, Zhou, *et al.*, 2007). Most of the studies on M2 receptor modulation of GABA and glycine release are related with the role of these receptors in nociception (Kurihara *et al.*, 1993; Gibson and Logan, 1995; Stewart and Maxwell, 2003; Wang *et al.*, 2006; H. M. Zhang, Zhou, *et al.*, 2007; Cai *et al.*, 2009;

Zhang *et al.*, 2009; Jeong *et al.*, 2013) and no direct relation was established with MN function. However, considering that M2 muscarinic receptors are involved in the inhibition of the monosynaptic reflex activated by nociceptive stimuli (Kurihara *et al.*, 1993) which might affect lumbar locomotor CPGs (Mandadia *et al.*, 2009) an eventual M2 receptor-mediated modulation of inhibitory inputs from dorsal INs to MNs cannot be discounted. This subtype of muscarinic receptor has also been shown to decrease glutamatergic transmission from primary sensory afferents to lamina II INs (H. M. Zhang, Chen, *et al.*, 2007; Jeong *et al.*, 2013) and muscarinic receptor activation decreased evoked AMPA-mediated synaptic currents recorded from mouse MNs after stimulation of the spinal cord dorsolateral funiculus (Mejia-Gervacio, 2012), suggesting that perhaps the observed decrease in synaptic drive could also reflect M2 muscarinic receptor actions on glutamatergic synapses. To fully address if activation of the M2 receptor exclusively affects excitatory and/or inhibitory PSCs, voltage-clamp experiments should be repeated with muscarine co-perfused with 4-DAMP (to activate exclusively M2 receptors) in the presence of strychnine and picrotoxin allowing the acquisition of excitatory PSCs. Conversely, clamping MNs at a holding potential of -40mV would enable isolation and investigation of inhibitory PSCs. Nevertheless, the results obtained indicate that M2 receptor activation decreased the overall synaptic drive to MNs.

5.1.4. Modulation of MN function by M3 muscarinic receptors

The M3 muscarinic receptor antagonist 4-DAMP has previously been shown to block ACh-mediated depolarizations in mouse MNs (Bertrand and Cazalets, 2011). In the present work activation of M3 muscarinic receptors elicited an inward current in

MNs from spinal cord slices which was accompanied by an increase in input resistance that had a reversal potential close to -80/-90mV. The M3 receptor antagonist itself decreased input resistance with the current-voltage linear regression showing a reversal around -83mV. All these values are relatively close to the reversal potential for K^+ in the solutions used (-98mV) suggesting that these receptors could modulate leak K^+ channels in MNs, as has been shown in corticocallosal neurons (Jones and Baughman, 1992) and sacral relay neurons (Zhu and Uhlrich, 1998). This would result in an increase in excitability by bringing the MN resting potential closer to the firing threshold. The M3 receptor-dependent inward current and increase in input resistance could also result from a modulation of other mechanisms, such as M-currents. In spinal MNs the M-current can be inactivated by M3 receptors (Bertrand and Cazalets, 2011) thus modulating the voltage threshold and up-regulating MN basal excitability (Lombardo and Harrington, 2016). Pharmacological activation of the channels responsible for the M-current can cause a hyperpolarization and decrease in input resistance whereas blockade can increase membrane resistance and depolarize MNs (Lombardo and Harrington, 2016). This could suggest that the observed inward current and increased input resistance as a result of M3 receptor modulation could reflect a negative regulation of the M-current in MNs. The use of XE991 to selectively block this particular current followed by M3 receptor activation could help to resolve whether some of the M3 muscarinic receptor effects on input resistance and holding current are partially a result of the modulation of the M-current.

Regarding MN output, activation of M3 muscarinic receptors in the presence of M2 antagonists had no significant effect on MN maximum output or mAHP amplitude. However, blockade of these receptors with 4-DAMP removed the muscarine-induced

increase in maximum firing suggesting that activation of M3 receptors is involved in cholinergic modulation of MN output. Very few studies have addressed the role of M3 muscarinic receptors in the modulation of MN output with the majority of observations within motor systems coming from studies performed in brainstem INs. In recordings from INs of the preBötzinger Complex involved in the generation of respiratory-related rhythmicity, 4-DAMP removed an increase in tonic firing elicited by raising the levels of ACh with ACh-esterase inhibitors (Shao and Feldman, 2000, 2005). This activation of muscarinic receptors in the preBötzinger Complex induced a current that was associated with an increase in input resistance and a reversal potential of -11.4mV. The authors suggested this reflected the opening of a non-selective channel permeable to both Na^+ and K^+ that is not Ca^{2+} activated (Shao and Feldman, 2000). This assumption contrasts with the I-V relationship results for the experiments performed in mouse MNs (reversal at -80/-90mV) indicating that M3 muscarinic receptor-modulation might be different amongst different subtypes of spinal and brainstem neurons.

One of the subsequent targets of M3 receptor modulation in neurons are M-channels which are K^+ channels exclusively modulated by ACh that generate a delayed rectifier current (M-current) which can limit action potential threshold and firing output (Brown and Passmore, 2009). In the central nervous system the M-current is exclusively modulated by M1 and M3 receptors (Brown and Passmore, 2009) and its modulation may affect MN function (Bertrand and Cazalets, 2011). In hippocampal pyramidal neurons this current supresses the duration and frequency of trains of spikes and blockade or genetic suppression of M-currents increases maximum firing and spike adaptation (Brown and Passmore, 2009). Considering that the M-current might be tonically active in lumbar MNs and can be downregulated by M3 muscarinic receptor

activation (Bertrand and Cazalets, 2011) it can be hypothesized that M3 receptors might be partially necessary for the increase in MN output elicited by muscarine. Perhaps activation of M3 receptors is followed by a subsequent downregulation of M-channels which will allow MNs to fire at higher frequencies in the presence of an excitatory stimuli, as has been reported for hippocampal synapses (Brown and Passmore, 2009). As discussed before, the I_h current can also be modulated by ACh in MNs (Chevallier *et al.*, 2006, 2008). In relay cells in the rat lateral geniculate nucleus, M3 muscarinic receptor activation increases I_h and decreases leak K^+ conductance resulting in increased neuronal excitability (Zhu and Uhlrich, 1998). Considering that M2 receptors downregulate I_h (Chevallier *et al.*, 2006; Zhao *et al.*, 2016), perhaps a balance between both M2 and M3 receptor activation is necessary for muscarine to increase MN output (section 5.1.5). Nonetheless, the results from this thesis show that M3 muscarinic receptors are involved in the muscarine-induced increase in MN excitability. Whether the M-current or other channels represent the target for M3 receptor regulation of MN output remains to be explored.

In experiments using electrical stimulation of the ventral commissure in spinal cord slices, high concentrations of 4-DAMP increased the amplitude and duration of postsynaptic potentials recorded from MNs indicating that M3 receptor activation decreases network excitation to MNs (Bertrand and Cazalets, 2011). In the current reported experiments, M3 receptor activation increased synaptic drive to MNs. As previously discussed, the acquired PSCs might comprise both excitatory and inhibitory currents. Somatodendritic M3 muscarinic receptors potentiate glycine release from lamina II INs (Wang *et al.*, 2006) and do not seem to modulate GABAergic release from the dorsal horn (Zhang *et al.*, 2009). In M3 knockout mice, oxotremorine (non-

selective mAChR antagonist) decreased the frequency of inhibitory inputs from lamina II spinal neurons (H. M. Zhang, Zhou, *et al.*, 2007) suggesting that M3 receptors modulate inhibitory synaptic drive in dorsal INs. Revealing of inhibitory PSCs at a holding voltage of -40mV would help to clarify an eventual involvement of GABA and/or glycine release in M3 muscarinic receptor-mediated modulation of synaptic inputs to MNs. Since M3 activation can increase excitation of motor networks which contribute to the generation of bursts of ventral root activity in the rat (Jordan *et al.*, 2014), it could be possible that the M3 subtype is responsible for an increase in excitatory input to MNs. Experiments with blockers of inhibitory transmission in the presence of methoctramine and muscarine to record excitatory PSCs would help to clarify the role of M3 receptors. Nevertheless, the data shows that M3 receptor activation increased overall network input to MNs.

5.1.5. M2 and M3 muscarinic receptors differently modulate spinal locomotor circuits

Voltage-clamp recordings performed in MNs from the neonatal mouse spinal cord have demonstrated opposing effects associated with the activation of M2 and M3 muscarinic receptors. Regarding synaptic inputs, M2 receptor activation decreased PSC frequency, whereas M3 receptor activation caused an increase in synaptic drive to MNs. M2 muscarinic receptors were responsible for a outward current and a decrease in input resistance while M3 receptors depolarized MNs and increased input resistance. Differences in the functional roles of M2 and M3 muscarinic receptors in spinal physiology have been highlighted previously. Stimulation of the saphenous nerve can evoke lumbar ventral root potentials that are potentiated by methoctramine and

supressed by 4-DAMP (Kurihara *et al.*, 1993). In the dorsal horn, M3 receptors are present at glycinergic INs and potentiate glycine release, while M2 receptors counteract the effects on synaptic glycine release to dorsal INs (Wang *et al.*, 2006). In M3 receptor knockout mice, oxotremorine decreased the frequency of inhibitory PSCs whereas in M2 receptor knockout animals it increased the frequency of these inhibitory inputs to lamina II INs (H. M. Zhang, Zhou, *et al.*, 2007). The opposing effects of M2 and M3 muscarine receptors have not only been reported in the spinal cord but also in other central synapses. In the cerebellum-projecting medial vestibular nucleus M2 muscarinic receptors inhibit glutamate release from vestibular afferents whereas M3 muscarinic receptors increase excitability of neurons projecting from this nucleus to the cerebellum (Zhu *et al.*, 2016). M2 muscarinic receptors were responsible for an inhibitory response of ACh in parafascicular neurons whereas an excitatory component was mediated by M3 receptors (Ye *et al.*, 2009). Formation of heterodimers has often been implicated in functional interactions between G-protein coupled receptors (Maggio *et al.*, 1999). For example, D2 dopamine receptors and A_{2A} adenosine receptors can form heterodimers that are involved in neuroplasticity in the basal ganglia and in neurogenerative diseases such as Parkinson's disease (K. Fuxe *et al.*, 2003; Kjell Fuxe *et al.*, 2005). The A_1 (inhibitory) and A_{2A} (excitatory) adenosine receptors can also form dimeric structures that may be responsible for functional crosstalk at several synapses prompting a balance between an adenosinergic status of excitation and inhibition (Sheth *et al.*, 2014), which can be dysfunctional in diseases involving spinal MNs such as ALS (Nascimento *et al.*, 2014, 2015). M2 and M3 muscarinic receptors can also cross-interact with each other and form heterodimeric receptors (Maggio *et al.*, 1999; Novi *et al.*, 2005; Goin and Nathanson, 2006; Clovis *et al.*, 2016). However, immunoreactivity for M3 and M2

muscarinic receptors in the spinal cord suggests that there might not be an overlap that could be indicative of heterodimerization, at least on the soma of MNs. M2 muscarinic receptors are preferentially clustered at the C-bouton synapse whereas the M3 receptor subtype was not present at this synapse but in fine cytoplasmic puncta in MNs (Wilson *et al.*, 2004). Some INs in laminas X, VII and VIII exhibited M3 receptor immunoreactivity (Wilson *et al.*, 2004) whereas M2 muscarinic receptors were mostly found in laminas II-III, IV-VI and lamina X (Stewart and Maxwell, 2003). This suggests that there is a likelihood of both receptors being expressed in synapses around the ventromedial area of the spinal cord where some CPG INs responsible for the generation of locomotor patterns are located. Whether M2/M3 heterodimers or functional crosstalk is involved in the modulation of spinal neurons, remains a possibility that could be addressed in the future. The possibility of protein-protein interactions between M2 and M3 receptors in the spinal cord could be explored using bioluminescence resonance energy transfer to study the molecular details and functional role of such possible oligomeric assembly (Pfleger *et al.*, 2006).

An interesting feature from the results obtained is the loss of muscarine-induced increase in MN maximum firing in the presence of 4-DAMP or methoctramine. Several studies suggested that some muscarinic receptor-dependent actions result from synergistic or a balanced activation of different receptors (Novi *et al.*, 2005; González *et al.*, 2011; Matsuyama *et al.*, 2013). For example, in recordings from lamina II INs, both M2 and M3 muscarinic receptors were shown to regulate glutamate release with M2 receptor modulating release from primary afferents while the M3 subtype regulated transmission in a group of glutamatergic INs in the spinal cord (H. Zhang *et al.*, 2007). In a different study, intrathecal administration of methoctramine or 4-DAMP in the rat

spinal cord decreased the animal's sexual behaviour, suggesting that blockade of any of the two receptors disrupts muscarinic receptor actions (Gómez-Martínez and Cueva-Rolón, 2009). Considering the contrasting role of M2 and M3 muscarinic receptors in the regulation of currents such as I_h (Chevallier *et al.*, 2006; Zhao *et al.*, 2016; Zhu *et al.*, 2016) and leak K^+ channels (Jones and Baughman, 1992; Zhu and Uhlrich, 1998; Chevallier *et al.*, 2006) and their different distribution on the surface of MNs but also on diverse spinal INs (Hellström *et al.*, 2003; Stewart and Maxwell, 2003; Wilson *et al.*, 2004), it could be assumed that the muscarine-induced increase in MN output is not a direct outcome of the activation of one type of muscarinic receptor but instead a result of a balance between M2 and M3 receptor actions.

Blocking M2 muscarinic receptors during NMDA, DA and 5-HT-induced locomotion decreased the variance of burst frequency and increased the duration of locomotor-related bursts. In contrast, M3 receptor antagonists disrupted the rhythmicity of the evoked bursts while also decreasing burst duration. These results indicate that M2 and M3 muscarinic receptors have contrasting effects on the CPG network and therefore suggest that ACh, which is released during locomotion, facilitates a balance between M2/M3 actions to ensure effectiveness of locomotor-related network output. M3 muscarinic receptors seem to be important in setting up adequate inter-burst intervals and thus avoiding formation of clusters of erratic bursts. The change between disorganized patterns of activity (e.g. multirhythm, diverse pattern) and continuously rhythmic activity relies on appropriate network excitability. Neuromodulators such as dopamine or 5-HT can shift disorganized bursts that were elicited at low doses into regular bursting when perfused at high concentrations (Sharples and Whelan, 2017). Regarding dopamine modulation, concentration-dependent sequential activation of

different receptor subtypes seemed to be responsible for this transition (Sharples *et al.*, 2015). Neuromodulation is largely influenced by network state, which could indicate that perhaps M3 muscarinic receptors are important in stabilizing the drug-induced rhythm by raising network excitability possibly through inactivation of the M-current in different CPG INs. On the other hand, M2 muscarinic receptors seem to be important in maintaining some degree of irregularity in the locomotor rhythm. By allowing burst events to be shorter (decreased duration) it could grant some degree of flexibility to the spinal network by allowing a modulator or a supraspinal stimulus to elicit a faster locomotor pace. Differences in M2 and M3 muscarinic receptor modulation of rhythmic outputs have not only been evidenced in the rat spinal cord (Jordan *et al.*, 2014) but also in peripheral synapses where M2 and M3 receptors differentially regulate the intestinal motor activity with the M2 subtype being responsible for the generation of rhythmic motor activity while M3 receptors control periodicity of the rhythm (Tanahashi *et al.*, 2013). In addition, synergistic responses to activation of both receptors is involved in the cholinergic excitation of intestinal motor activity (Matsuyama *et al.*, 2013). Based on the above discussion one can assume that a balanced M2/M3 muscarinic receptor activation adequately regulates episodic bursting during generated by spinal locomotor networks.

5.2. Manipulation of Pitx2^{+} INs activity with DREADDs and impact on MN output and spinal locomotor networks

No work has been published yet on the expression of either excitatory or inhibitory DEADD receptors in genetically defined subsets of spinal INs that are part of the mammalian locomotor CPG. Considering this, the efficiency of DREADD

expression and impact on neuronal physiology will first be discussed and compared with reports using DREADD manipulation in other central systems. In CA1 pyramidal neurons expressing viral-injected hM3Dq, CNO increased firing frequency and recurrent bursting (Alexander *et al.*, 2009). Similarly, in agouti-related protein neurons from the hypothalamus, Cre-dependent expression of this receptor elicited rapid depolarization of the membrane potential and increased firing rates (Krashes *et al.*, 2011). In hippocampal neurons activation of the genetically inserted inhibitory hM4Di receptor decreased firing output (Zhu *et al.*, 2014) and was able to silence the occurrence of spontaneous action potentials (Armbruster *et al.*, 2007). In the present study, activation and inhibition of $Pitx2^{+}$ INs was achieved in Pitx2-Cre;TdTom;CHRM3 and Pitx2-Cre;TdTom;CHRM4 animals since spontaneous firing from these cells increased or decreased in the presence of CNO, respectively. Some constitutive actions of DREADD receptors have been reported recently (Armbruster *et al.*, 2007; Sheikhbahaei *et al.*, 2018), which could influence their use for the activation/inhibition of $Pitx2^{+}$ INs. $Pitx2^{+}$ INs are tonically active at rest at a frequency of approximately 3Hz (Zagoraiou *et al.*, 2009). In the current work it is reported that $Pitx2^{+}$ INs exhibited spontaneous activity at approximately 1.8Hz and 1.6 Hz in Pitx2-Cre;TdTom;CHRM3 and Pitx2-Cre;TdTom;CHRM4 mice, respectively, while in controls it as close to 1Hz. The reported values for $Pitx2^{+}$ INs spontaneous firing are lower when compared to the means (3Hz) previously reported (Zagoraiou *et al.*, 2009) probably because these recordings were performed in spinal cord hemisections where synaptic drive and neuronal arborization is more preserved than in the circuitry from spinal cord slices used in this work. Despite the spontaneous firing frequency being slightly higher in $Pitx2^{+}$ INs from DREADD than control animals, these average values

are not statistically different from each other which suggests the absence of a constitutive action of DREADDs on Pitx2^{+} INs. In addition, if a constitutive action from DREADDs was to be noticed, changes in MN maximum firing would be present, since Pitx2^{+} IN activation or inhibition significantly modulated MN output. When comparing MN maximum firing before CNO perfusion, both excitatory and inhibitory DREADD and control mice exhibit similar maximum spike frequency (approximately 25/26Hz). Due to the small evidence of constitutive DREADD activity in the vast repertoire of Cre-lox DREADD recombinations used (Roth, 2016; Zhu *et al.*, 2017) and the similarity in firing properties of Pitx2^{+} INs and MNs in DREADD and control mice before perfusion of CNO, a constitutive action of DREADDs on Pitx2^{+} INs was deemed improbable in the currently reported data.

The expression of DREADD receptors in the animals used in this work should be consistent with the pattern of expression of the Cre driver (Zhu *et al.*, 2017). Cre-dependent expression of fluorescent reporters in Pitx2^{+} INs was observed in more than 90% of cholinergic Pitx2^{+} INs and more than half of the glutamatergic Pitx2^{+} INs (Zagoraiou *et al.*, 2009). Immunohistochemical studies to address the percentage of Pitx2^{+} INs that express hM3Dq or hM4Di were not performed, however is it likely that the expression of DREADD receptors was similar to the high expression observed for fluorescent reporters using the same Pitx2-Cre driver line (Zagoraiou *et al.*, 2009; Zhu *et al.*, 2017).

5.2.1. Activation of Pitx2$^+$ INs increases MN output via M2 muscarinic receptor-dependent modulation of Kv2.1 channels

The activation of Pitx2$^+$ INs in Pitx2-Cre;CHRM3 mice induced a small, methoctramine-sensitive, inward current in MNs that was accompanied by an increase in input resistance and had a reversal potential of approximately -80mV. An increase in input resistance with a similar value for reversal was also observed in a group of MNs from WT mice after M2 muscarinic receptor activation. However, in these WT animals, activation of M2 muscarinic receptors induced an outward current as opposite of the Pitx2$^+$ IN-mediated inward current, indicating that overall M2 receptor modulation in WT mice involves contribution from other sources of cholinergic inputs that are not Pitx2$^+$. It is known that the expression of M2 muscarinic receptors is more prominent on large compared to small MNs, with particular clustering on MN areas juxtaposed to C-boutons (Welton *et al.*, 1999; Wilson *et al.*, 2004). The abundance of C-boutons does seem to vary between different MN pools. For example, MNs innervating groups of fast-twitch fibres within the medial gastrocnemius muscle have more C-boutons than those innervating the slow-twitch soleus muscle (Hellström *et al.*, 2003). Since fast-twitch fibres are innervated by large alpha MNs whereas slow-twitch muscle fibres receive projections from smaller alpha MNs, Pitx2$^+$ INs could have an important role in the size principle of motor unit recruitment (Henneman *et al.*, 1965; Llewellyn *et al.*, 2010). Due to the heterogenous anatomical distribution of C-boutons to different MN pools (Welton *et al.*, 1999; Hellström *et al.*, 2003; Wilson *et al.*, 2004) perhaps the increase in input resistance in MNs from WT mice after M2 receptor activation could be a reflection of a modulation involving Pitx2$^+$ INs. Perhaps these MNs have more C-boutons than other groups of MNs in which a decrease in input resistance was observed

when M2 receptors were activated. This could be clarified in the future with experiments addressing modulation of MN function by: (1) studying changes in input resistance after activating M2 receptors but in spinal cord slices of Pitx2-Cre;DTA mice in which C-boutons are ablated or (2) retrogradely label fast (gastrocnemius) or slow twitch (soleus) muscles that are differently innervated by Pitx2-INs (Hellström *et al.*, 2003) and record M2 receptor responses from the labelled MNs. When comparing results regarding M2 receptor-mediated modulation of subthreshold properties from WT and excitatory DREADD mice, it appears that the effects of Pitx2^{+} INs and C-bouton synapses may only account for a small portion of the overall M2 muscarinic receptor-mediated modulation of input resistance and current observed in WT mice. Experiments from Pitx2-Cre;CHRM3 mice indicate that activation of Pitx2^{+} INs increases cell excitability by depolarizing MNs and increasing input resistance through a M2 receptor-dependent mechanism that does not involve Kv2.1 channels, but perhaps includes other proteins that are expressed at the postsynaptic terminal opposite C-boutons and that might ultimately influence MN output (Witts *et al.*, 2014).

In Pitx2-Cre;CHRM3 mice activation of the hM3Dq receptor, and hence activation of Pitx2^{+} INs, with CNO increased MN firing. This effect was eliminated by a M2 muscarinic receptor antagonist or with prior blockade of Kv2.1 channels. M2 muscarinic receptors are clustered on MN soma juxtaposed to C-boutons and have previously been implicated in modulation of motor output by Pitx2^{+} INs (Hellström *et al.*, 2003; Wilson *et al.*, 2004; Miles *et al.*, 2007; Zagoraiou *et al.*, 2009; Witts *et al.*, 2014). By stimulating Pitx2^{+} INs while recording MN output, the current study provides the first direct evidence that M2 receptors contribute to muscarinic modulation of spinal locomotor output. Several proteins, which are clustered near M2 muscarinic receptors at

the C-bouton synapse such as SK channels, Kv2.1 channels and N-type Ca^{2+} channels, could be downstream targets of M2 receptors for subsequent regulation (Witts *et al.*, 2014). Previous experiments hypothesised that SK channels are subject to M2 muscarinic receptor-mediated modulation at C-boutons leading to a decrease in mAHP amplitude which could translate into increased MN excitability (Miles *et al.*, 2007). However, in the current study, DREADD-mediated activation of $Pitx2^{+}$ INs resulted in an increase in the mAHP in MNs which does not match previous suggestions (Miles *et al.*, 2007). Experiments performed in WT mice revealed that a muscarine-induced decrease in mAHP was dependent on M2 receptor activation. Perhaps M2 muscarinic receptors that are also known to be present outside the C-bouton synapse (Wilson *et al.*, 2004) could be responsible for the decrease in mAHP amplitude in WT mice. N-type Ca^{2+} channels can also modulate mAHP kinetics and affect SK-mediated hyperpolarizations. Ca^{2+} entry through N-type Ca^{2+} channels after an action potential can activate SK channels leading to an increase in the mAHP (Hallworth *et al.*, 2003; Kasten *et al.*, 2007; Vrindab *et al.*, 2016). SK-mediated currents have been shown to be activated by N-type Ca^{2+} channels in rat MNs (Li and Bennett, 2007). Perhaps activation of $Pitx2^{+}$ INs could increase mAHP amplitude by indirect regulation of an SK-dependent AHP through modulation of N-type Ca^{2+} channels that are known to be positioned on MN somata and terminals at C-bouton synapses and might increase Ca^{2+} influx to activate SK channels. In dorsal root ganglia neurons, blockade of Kv2.1 channels decreases mAHP amplitude (Tsantoulas *et al.*, 2014), suggesting that a functional up-regulation of Kv2.1 channels could be responsible for changes in the MN mAHP. However, a role for Kv2.1 channels in the regulation of the mAHP in MNs is very unlikely since mAHP amplitude was still increased following activation of $Pitx2^{+}$

INs in the presence of the Kv2.1 channel blocker guangxitoxin-1E. Therefore, the increase in mAHP amplitude involves M2 muscarinic receptor-mediated modulation that does not target Kv2.1 channels. The channels involved and the molecular pathways underlying changes in the amplitude of the mAHP in MNs as a result of activation of $Pitx2^{+}$ INs in Pitx2-Cre;CHRM3 mice remain to be fully determined.

The current study revealed a decrease in the voltage threshold for action potential generation after activation of $Pitx2^{+}$ INs in Pitx2-Cre;CHRM3 mice. The decrease in threshold was associated with M2 muscarinic receptors but not of Kv2.1 channels, suggesting that other proteins present at the C-bouton synapse, such as SK and N-type Ca^{2+} channels, could be responsible for the reported change in action potential threshold. In subthalamic nucleus neurons inhibition of N-type Ca^{2+} channels in the absence of SK channel activity resulted in an increase in action potential threshold (Hallworth *et al.*, 2003), while SK channel blockade depolarized the action potential threshold in midbrain dopamine neurons (Iyer *et al.*, 2017). Whether M2 muscarinic receptors regulate the action potential threshold of MNs via actions on SK or N-type Ca^{2+} channels at the C-bouton synapse could be addressed in future work. By decreasing the voltage threshold for action potentials, $Pitx2^{+}$ INs decrease the current necessary from an incoming stimulus to trigger MN firing which in turn translates into amplified motor unit excitability.

Increased MN spiking after the activation of $Pitx2^{+}$ INs was dependent on Kv2.1 channels, and their regulation of the half-width of evoked action potentials. Kv2.1 channels are not only expressed at the C-bouton synapse but also at other synaptic sites on MNs associated with contacts from presumptive excitatory INs (Muennich and Fyffe, 2004; Fletcher *et al.*, 2017). Kv2.1 currents enable repetitive firing in a wide

range of neuronal types (Malin and Nerbonne, 2002; Guan *et al.*, 2013; Liu and Bean, 2014), playing an important role in the repolarization phase of action potentials in MNs (Gao and Ziskind-Conhaim, 1998). The reduction in MN spike frequency observed in a mouse model of Spinal Muscular Atrophy has recently been attributed to a reduction in Kv2.1 currents, which lead to an increase in the half-with of MN action potentials thus limiting firing output (Fletcher *et al.*, 2017). By increasing K^+ conductance through modulation of Kv2.1 channels, M2 muscarinic receptors at the C-bouton synapse allow for shorter duration action potentials, which translates into increased repetitive firing. This feature seems to be exclusively mediated by Kv2.1 channels and probably independent from other proteins at the C-bouton synapse. Modulation of SK channels has been shown to affect mAHP and not action potential duration (Gao and Ziskind-Conhaim, 1998) while N-type channel blockers did not change the half-width of action potentials in hippocampal pyramidal neurons (Shah and Haylett, 2000).

Kv2.1 channels can be subjected to different modulatory influences including phosphorylation (Mohapatra, 2006). In hippocampal neurons muscarinic stimulation via M1 or M3 receptors increased calcineurin-meditated dephosphorylation of Kv2.1 channels, which reduced their activity (Mohapatra *et al.*, 2007). Authors also found that the C-terminus of Kv2.1 channels can act as a transferable "muscarinic response element" conferring muscarinic modulation of function and location (Mohapatra *et al.*, 2007). M2 muscarinic receptor activation can lead to subsequent activation of several phosphorylation pathways (Shapiro *et al.*, 1999), which could ultimately regulate Kv2.1 channel phosphorylation at C-bouton synapses thus increasing MN firing (Muennich and Fyffe, 2004).

The results reported here provide direct evidence that Pitx2$^+$ INs modulate MN output in the neonatal mouse spinal cord. The lack of evidence of anything else than a cholinergic modulation from Pitx2$^+$ INs in DREADD mice supports a role for C-boutons in the modulation of MN output. However, since some Pitx2$^+$ INs are glutamatergic and their contribution to MN function perhaps through polysynaptic pathways that might affect dorsal cholinergic INs that might modulate MN output remains a possibility open to discussion. In sum, the data show that Pitx2$^+$ INs modulate MN function through the activation of M2 muscarinic receptors at the C-bouton synapse, which increase firing output by reducing action potential duration through regulation of Kv2.1 currents.

5.2.2. Pitx2$^+$ INs influence the strength of motor output during locomotion

As discussed previously, a constitutive action of hM4Di on Pitx2-Cre;CHRM4 mice was excluded, therefore the results indicate that activation of the DREADD receptor with CNO was able to effectively inhibit Pitx2$^+$ INs. This cholinergic subset of INs is active during fictive locomotion exhibiting bursts of spikes that are in phase with segmentally aligned ventral root output (Zagoraiou *et al.*, 2009). CNO-induced inhibition of Pitx2$^+$ INs elicited a M2 receptor-dependent outward current in MNs that was associated with a decrease in input resistance and had a reversal potential close to the equilibrium potential for K^+ in the solutions used (-98mV). This could indicate that removing the input from Pitx2$^+$ INs to MNs during drug-induced locomotion contributed to a reduction in MN excitability due to MNs becoming more hyperpolarized following the opening of leak K^+ channels. The effects of reducing MN

excitability in Pitx2-Cre;CHRM4 would lead to a decrease in MN spiking in the presence of locomotor drugs. This indicates that activation of Pitx2^{+} INs during locomotion might change the intrinsic properties of MNs rendering them capable of firing at higher frequencies during NMDA, 5-HT and DA-induced locomotion.

The decrease in MN output observed in Pitx2-Cre;CHRM4 mice resulted in an reduction in ventral root burst amplitude suggesting that the prominent role of this subpopulation of INs during fictive bursting is the fine-tuning of the strength of motor output. Despite there being some evidence of connectivity between Pitx2^{+} INs and other spinal INs, with glutamatergic Pitx2^{+} INs projecting to dorsal INs and a low density of Pitx2^{+} cholinergic boutons observed in the intermediate zone of the spinal cord (Zagoraiou *et al.*, 2009), neither ventral root burst frequency or duration changed with CNO application in Pitx2-Cre;CHRM4 mice. Since the effects of inhibition of Pitx2^{+} INs were dependent on M2 muscarinic receptor activation and no changes in the frequency or duration of locomotor-related bursts were observed, data from experiments involving DREADD-mediated inhibition of Pitx2^{+} INs strongly support a primary role for Pitx2^{+} INs and C-boutons in the control of MN output with little or no impact on CPG INs.

Pitx2^{+} INs are active enhancers of MN output during drug-induced locomotion. Silencing these INs reduced MN excitability resulting in decreased ventral root motor output. The fact that methoctramine blocked the effects of DREADD-mediated inhibition of Pitx2^{+} INs indicates that modulation by M2 muscarinic receptors at the C-bouton synapse is involved, as seen in experiments utilising Pitx2-Cre;CHRM3 animals for DREADD-mediated excitation of Pitx2^{+} INs. The possibility of other modulatory effects on motor control being attributable to this particular subset of cholinergic INs

could be explored in different experimental settings. For example, future experiments could utilise DREADDs to manipulate Pitx2^{+} IN activity during sensory-evoked motor activity in isolated spinal cords, brainstem stimulation in intact animals, or during behavioural tests in adult animals, such as reaching tasks and rota-rod tests. Such experiments would help determine if the modulatory roles of Pitx2^{+} INs extend beyond control of MN output during locomotion.

5.3. Genetic ablation of cholinergic Pitx2^{+} INs removes M2 receptor-dependent modulation of the amplitude of motor output during locomotion

Cre-dependent expression of diphtheria toxin A has been widely used to genetically ablate specific populations in the spinal cord to study locomotor networks involved in the control of hindlimb movement (Gosgnach *et al.*, 2006; Crone *et al.*, 2008). Pitx2-Cre;DTA mice exhibit 90% less C-boutons than control mice, thus illustrating the high degree of efficiency in the genetic removal of cholinergic Pitx2^{+} INs in this animal model. Results from WT mice indicated that blocking M2 muscarinic receptors with methoctramine reduced burst amplitude, while experiments utilising DREADDs to activate Pitx2^{+} INs showed that M2 receptors are responsible for the regulation of MN output at the C-bouton synapse. Removal of cholinergic Pitx2^{+} INs allowed for further investigation of the physiological relevance of these neurons in spinal circuits. Previous work showed that elimination of cholinergic Dbx-1 expressing INs, which includes ACh-expressing Pitx2^{+} INs, did not affect the viability of mice nor general locomotor pattern (Zagoraiou *et al.*, 2009). Therefore we would expect that

ablation of cholinergic Pitx2^{+} neurons in Pitx2-Cre;DTA mice would not produce overt phenotypes in mice. The Pitx2 transcription factor is expressed in the dorsal and ventral midbrain and may be important in the differentiation of restricted GABAergic and glutamatergic populations of neurons (Waite *et al.*, 2011). However, it is not known if cholinergic Pitx2^{+} INs are present in the brain and if they impact motor function. Although, no specific tests were performed to ascertain if Pitx2-Cre;DTA mice exhibited changes in development, growth or general locomotion, no overt motor phenotypes were observed in these mice. It is possible that compensatory mechanisms might operate to reduce the effect of the loss of cholinergic Pitx2^{+} INs. Such compensation has been reported during embryonic spinal cord development in mice lacking ChAT enzyme, although these mice did exhibit a partial lack of locomotor fidelity (Myers *et al.*, 2005). Experiments in the current study demonstrated that isolated neonatal spinal cords obtained from Pitx2-Cre;DTA mice were viable and exhibited regular bursts of locomotor activity. Since methoctramine did not affect the amplitude of ventral root bursting in Pitx2-Cre;DTA spinal cords, results further highlighted the role of M2 muscarinic receptors at C-bouton synapses in the modulation of the strength of motor output.

Adjusting the amplitude of motor output during locomotion will help to match the strength of muscle contraction to behavioural demands. Pitx2^{+} INs appear to be part of an intrinsic spinal cholinergic modulatory system that regulates the strength of the motor output during coordinated locomotion. Previous work suggested that recruitment of this modulation occurs in a task-dependent fashion (Zagoraiou *et al.*, 2009) and therefore descending or sensory inputs that could relate to motor commands such as posture control (Stepien *et al.*, 2010; Murray *et al.*, 2018) could trigger activation of

Pitx2^{+} INs which would in turn release ACh at the C-bouton synapses thus increasing MN firing through M2 receptor-dependant mechanisms (Miles *et al.*, 2007; Zagoraiou *et al.*, 2009; Witts *et al.*, 2014). Beyond this clear role for the Pitx2^{+} IN system in the regulation of the intensity of motor output during hindlimb locomotion, future behavioural analyses of Pitx2-Cre;DTA animals will be important to determine whether the Pitx2^{+} IN system is also involved in additional, possibly more subtle modulation of motor control.

6. CONCLUDING REMARKS

The work described in this thesis explored how muscarinic receptors affect motor output and MN function in the lumbar region of the neonatal mouse spinal cord. Using electrophysiological recordings of MNs in isolated spinal cord preparations, along with ventral root recordings during drug-induced locomotion, it was possible to elucidate the cellular mechanisms underlying M2 and M3 muscarinic receptor modulation of spinal excitability and the involvement of these receptors in circuits responsible for the generation of rhythmic patterns of locomotor activity. Several previous studies have described some of the roles of muscarinic receptors in the control of spinal motor function in different vertebrates such as the salamander (Alaburda *et al.*, 2002; Chevallier *et al.*, 2006, 2008), turtle (Alaburda *et al.*, 2002), rat (Kiehn *et al.*, 1996; Jordan *et al.*, 2014; Anglister *et al.*, 2017) and mouse (Jiang *et al.*, 1999; Miles *et al.*, 2007). Despite the shared commonalities between observations, there are often discrepancies, even when comparing close species such as rat and mice (Kiehn *et al.*, 1996; Jiang *et al.*, 1999). The current study sought to clarify the role of cholinergic modulation in the neonatal mouse spinal cord. In addition, this study focussed on a particular subpopulation of cholinergic INs – $Pitx2^{+}$ INs because they are best characterized and the only genetically traceable group of cholinergic INs in the spinal cord. Use of state-of-art genetic tools allowed detailed exploration of muscarinic modulation mediated by $Pitx2^{+}$ INs.

This study demonstrated that M2 and M3 muscarinic receptors are solely responsible for muscarinic modulation of spinal locomotor output. A balance between M2 and M3 receptor activation is involved in sustained rhythmic fictive locomotion. M3 muscarinic receptors seem to be important for stabilizing the locomotor rhythm, whereas activation of M2 muscarinic receptors increases irregularity in the frequency of

locomotor bursts but also increases the strength of ventral root bursting by increasing MN output during locomotion. Experiments investigating the role of M2 and M3 receptors in the modulation of MN function suggest that these receptors play opposing roles. Both M2 and M3 receptors are present at last-order synapses to MNs. M2 muscarinic receptors decrease synaptic drive and hyperpolarize MNs due to the opening of leak K^+ channels. M3 receptors on the other hand, increase synaptic inputs to MNs and elicit a depolarization which could be due to regulation of a ionic mechanisms such as the M-current (Alaburda *et al.*, 2002; Bertrand and Cazalets, 2011; Lombardo and Harrington, 2016). M3 muscarinic receptor activation dominates in larger MNs, whereas M2 muscarinic receptor-mediated responses dominate in smaller MNs based on the capacitance values recorded. Interestingly both receptors are involved in the muscarine-elicited increase in MN output indicating that the disruption of the M2/M3 receptor balance might have consequences for cholinergic modulation of motor output. The current work has demonstrated that M2 and M3 muscarinic receptors have complementing effects on the modulation of MN function and spinal networks involved in the control of mammalian hindlimb locomotion.

Experiments investigating the roles of specific subtypes of cholinergic INs, which focussed on $Pitx2^+$ INs, defined cellular mechanisms involved in cholinergic control of motor output. In the lumbar spinal cord of the neonatal mouse, activation of $Pitx2^+$ INs during locomotion causes release of ACh from C-boutons that activate postsynaptic M2 muscarinic receptors, which in turn regulate the activity of Kv2.1 channels allowing for a faster repolarization phase during an action potential, resulting in increased MN firing and subsequent increased muscle contraction (figure 5). DREADD-mediated inhibition of $Pitx2^+$ INs reduced motor output during locomotion,

while genetic ablation of cholinergic Pitx2^{+} INs removed any M2 muscarinic receptor-mediated effects on the amplitude of locomotor bursts, proving that this population of cholinergic cells is important for the adjustment of the strength of motor activity. This information not only advances our understanding of the networks involved in hindlimb locomotion but also characterizes a group of important modulatory spinal INs that is severely affected in spinal cord injury and ALS.

In summary, this work has provided valuable novel information regarding cholinergic modulation of mammalian spinal locomotor circuitry. Receptors involved in muscarinic modulation have been pharmacologically characterized and their mechanisms of action explored. A particular subset of cholinergic neurons - Pitx2^{+} INs – has been found to contribute to this modulation by increasing MN output and therefore enhancing muscle contraction. In the future, anatomical and behavioural studies should be performed to fully address the anatomical distribution of M2 and M3 receptor modulation and the impact that DREADD-mediated modulation of Pitx2^{+} INs might have *in vivo*.

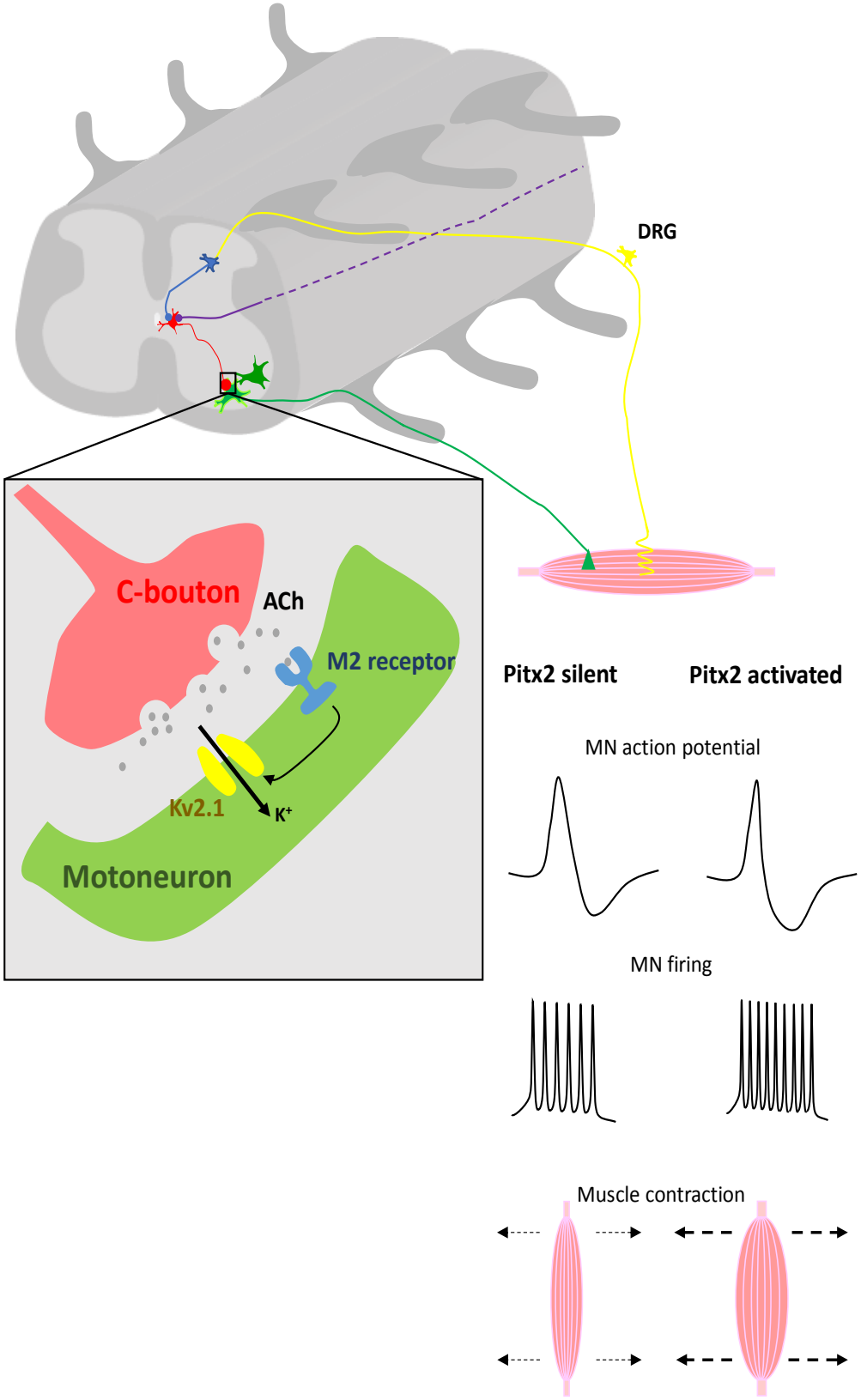
DRG
C-bouton
ACh
M2 receptor
Kv2.1
K+
Motoneuron
Pitx2 silent
Pitx2 activated
MN action potential
MN firing
Muscle contraction

Figure 5 – Suggested mechanism of Pitx2^{+} IN-mediated increase in motor output. Pitx2^{+} INs (red) form C-bouton synapses on MNs (green) and are known to received disynaptic inputs from sensory afferents (blue) as well as input from higher brain areas (purple) (Zagoraiou *et al.*, 2009). Activation of Pitx2^{+} INs will activate postsynaptic M2 muscarinic receptors at the C-bouton synapse that will positively regulate Kv2.1 channels. This modulation leads to a decrease in action potential duration, allowing for increased MN firing output which will translate into more intense contraction of muscle; DRG – dorsal root ganglion.

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